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Several ANK repeat-containing proteins have been implicated in breast cancer and other tumor development. Our purpose has been to define the function of the ANK repeats using a combination of genetics and biochemistry. This involved exhaustively mutagenizing the ANK repeats of the yeast Swi6 protein in order to identify the critical residues within each repeat. The effects of these mutations upon DNA binding and transcriptional activity was determined. Partially defective mutants have been identified and used to search for interacting gene products using suppressor analysis. We have concluded that ANK repeats play a structural role. ANK repeats represent one way to stably fold a polypeptide chain, and this structure provides a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues within the repeats are responsible for the specific protein-protein interactions of each class of ANK repeat proteins. From this we conclude that the right path to understanding the functions of the oncogenic repeat proteins is to focus upon the nonconserved, surface residues within their ANK repeats and search for proteins which interact with them. This work and the available Xray data provide a straight forward path to this goal.

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Linda Breeden 2 August 99

PI - Signature Date

The Role of the ankyrin repeats in the Swi4/Swi6 transcription complex of budding yeast.

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INTRODUCTION

Over 100 proteins have been identified as containing ankyrin repeats. Several proteins have received particular interest because of potential roles for the ankyrin repeat motifs in tumor development. These include Bcl-3, *int*-3, TAN-1, and p16. The *BCL-3* gene encodes a member of the I kappa B family of proteins and rearrangements involving the ankyrin repeat region *BCL-3* have been identified in B-cell chronic lymphocytic leukemias [1]. *Int-3*, a mouse proto-oncogene, is a common insertion site for the Mouse Mammary Tumor Virus (MMTV). Activation of the *int-3* ankyrin repeat sequences by MMTV produces poorly differentiated adenocarcinoma of the mammary and salivary glands in mice [2]. *TAN-1*, the human *Notch* homologue, was first described as a breakpoint region of t (7,9) gene rearrangements found in T-cell acute lymphoblastic leukemias. This rearrangement places the gene encoding the beta T-cell receptor adjacent to the ankyrin repeat motifs of *TAN-1* [3]. p16 is the protein product of the *MTS1* (multiple tumor suppressor-1) gene. It is composed of only a series of four ankyrin repeats and is one member of the cyclin dependent kinase inhibitor family of proteins. p16 can compete with cyclin D and displace the cyclin subunit from its kinase binding site, thereby inhibiting G1 to S progression through the cell cycle. Mutations in p16 have been noted in a variety of human tumors and tumor cell lines, including bladder and gastrointestinal neoplasms and mesotheliomas; in addition, mutations have been found in the germline of families predisposed to melanoma [4]. Ankyrin repeats were originally described by Breeden and Nasmyth [5] in the Swi6 transcription activator of the budding yeast, *Saccharomyces cerevisiae*. Swi6, with its two known protein binding partners, Swi4 and Mbp1, regulates gene expression during the G1 to S transition of the yeast cell cycle. All three of these proteins contain ankyrin repeats and the goal of this research was to understand the role of these repeats using a combination of genetics and biochemistry.

BODY

Our purpose has been to define the function of the ANK repeats. This involved exhaustively mutagenizing the ANK repeats of Swi6 to identify the critical residues within each repeat. The effects of these mutations upon DNA binding and transcriptional activity was determined. Partially defective mutants have been identified and used to search for interacting gene products using suppressor analysis. The results of these experiments has led us to the view that most of the ANK repeat domain has a structural role, and to consider alternate strategies for identifying surfaces of the ANK repeat motif which might bind to other proteins.

The technical objectives in our approved Statement of Work are to:

1. Generate monoclonal and polyclonal antibodies that recognize either Swi4, Swi6 or all ANK repeats and use them to screen for other ANK repeat-containing proteins.

2. Exhaustively mutagenize the ANK repeats of Swi4 and Swi6, and identify the crucial residues for their activity.

3. Develop a battery of genetic screens to identify mutations that cause loss or deregulation of known Swi4 and Swi6 functions.

4. Perform *in vitro* assays to correlate mutant phenotypes with known biochemical functions.

5. Identify second site suppressors, either within the Swi protein, or within associated proteins.

TASK 1. Generation of antibodies to the Swi6 ANK domain.

We have generated new polyclonal and monoclonal antibodies to the Swi6 protein. The polyclonals are very high quality and can be used at a dilution of 1:10,000 on westerns. These have been valuable reagents for isolating and analyzing swi6 mutants levels. The technical difficulties associated with obtaining large quantities of Swi4 protein have precluded the making of more Swi4 antibodies of any kind. Thus, we have focused our efforts upon understanding the ANK domain function of the Swi6 protein only. The Swi6 protein was purified as described [6] and the polyclonal serum was generated in rabbits with three subcutaneous injections of 100 micrograms of antigen in Freunds adjuvant following standard protocols [7]. Milligram quantities of Swi6 was also purified by Bernard Mai to facilitate chrystalization trials, which were unsuccessful.

Our monoclonal antibodies were generated by Dr. Elizabeth Wayner and Davis Creemer. Their respective reacitivities with Swi6 on Elisa, Western and immunoprecipitations (IP) are summarized in Table 1. To generate these monoclonal cell lines, eight week old RBF/DnJ [or BALB/cByJ(8.12)5Bnr/J] mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were given multiple injections of Swi6 protein or ankyrin domain fragment (50-100 μg to immunize and 25 -50 μg to boost) emulsified in complete or incomplete Freund's adjuvant. Complete Freund's adjuvant was used only to immunize naive mice. All emulsifications were prepared as a ratio of 60% oil to 40% aqueous (PBS). Swi6 specific antibody titers in the polyclonal antiserum were determined by ELISA and Western blot. Mice were selected for fusion based on specific antibody titers to Swi6. Monoclonal antibodies to Swi6 were produced as previously described [8]. Briefly, spleen cells from the immunized mice were fused with NS-1/FOX-NY myeloma cells [9]. Viable heterokaryons were selected in RPMI 1640 medium supplemented with adenine/aminopterin/thymidine [10]. Cultures secreting antibody specific for Swi6 were identified by ELISA using an HRP-conjugated goat antibody (Roche Molecular Biochemicals, Indianapolis, IN) specific for mouse antibodies with an IgG-type heavy chain. Appropriate antibody cultures were further identified by Western blot . Monoclonal hybridoma cell lines secreting antibodies with the desired specificity were produced via two rounds of cloning via limiting dilution.

One obstacle to characterizing all of these monoclonal antibodies in more detail was the fact that the antibody titer is too low in culture supernatants to be useful. To get around this we have generated ascities fluid containing a subset of these antibodies. Large-scale mAb production was undertaken by ascites production using stable hybridoma cell lines injected into athymic nu/nu mice. Isotypes of resulting mAbs were determined by using Isostrip Mouse Monoclonal Antibody Isotyping Kits (Roche). There are 23positive monoclonal cell lines in all, most of which have been generated with the isolated ANK domain. These are useful reagents for detecting Swi6 on Westerns , but they do not recognize any other ankyrin repeat containing proteins and thus are not recognizing epitopes common to other ANK domains. Thus they have not been useful in studying the structure or activities associated with ANK domains generally. However, these cell lines have been saved and will be useful for further studies of Swi6.

Table 1.Swi6 Ankyrin Repeat Domain Monoclonal Antibodies

	ELISA	Western	I.P.
1.2.C6-C11	√	√	
1.2.D10-B6	√	√	
1.2.E2-A6	√		
1.4.D1-H3	√	√	
1.4.D9-D6	√	√	√
1.1.E5-B5	√	√	
1.4.F12	√	√	
1.4.F8	√	√	
1.6.D1	√	√	
1.6.D8	√	√	
2.1.C7	√	√	
2.1.D3	√	√	
2.1.G3	√	√	
2.1.G4	√	√	
2.3.E10	√	√	
2.3.F2	√	√	
2.3.G6	√	√	
2.4.A5	√	√	
2.4.B9	√	√	
2.4.E9	√	√	

Swi6 (full length) Monoclonal Antibodies

	ELISA	Western	I.P.
4.1.G1	√	√	
4.1.A12	√	√	
4.4.F12	√	√	√

Table 1. The names and properties of the 23 monoclonal cell lines derived in this study are listed. A check mark indicates that supernatant and/or ascites fluid containing these antibodies react positively by ELISA, Western or immunoprecipitation (I.P.) assays against Swi6 as described in Appendices 1 and 2. Positions left unchecked indicate that this line has not been assayed for this activity.

TASK2. Define critical amino acids involved in ANK repeat function.

We have constructed a total of four libraries of *SWI6* genes with randomly mutagenized ANK repeat regions. We have isolated about 30 different temperature sensitive (ts) and 110 unconditionally inactive (null) Swi6 ANK mutants. Since most of the null mutants appeared to have lost expression of Swi6, we concentrated our efforts on ts mutations, the majority of which expressed Swi6. All of the temperature sensitive mutants are listed in Appendix 1, Figure 2. This panel of mutations demonstrates that mutations occur throughout the region and are about as likely to occur in any of the four ANK repeats . There is some clustering at each end of the whole ANK domain (although this could be a PCR artifact). The only region lacking mutations is the non-structured part of the spacer. Thus, all four ANK repeats are critical for Swi6 function.

We have identified five critical residues: R344, G347, D375, A477 and N500 at which single substitutions are enough to compromise the activity of the whole protein. These are marked as solid ellipses in Figure 1. Most, if not all, of the mutants that we looked at were defective both in SCB driven transcription and in MCB driven transcription (see Task 3).

We have also performed a screen for mutations that will hyperactivate the ANK repeat. We used one of the libraries that was most heavily mutagenized. No ANK mutations with hyperactivating characteristics were found. However, on two occasions we identified rare recombination products that resulted in duplication of the *HIS3* gene itself, which led to increased *HIS3* expression from an *MCB:HIS3* reporter. This suggested to us that our screening strategy (for elevated *HIS3* expression using the growth advantage on aminotriazole) had worked. It is therefore likely that a hyperactivating mutation cannot occur in the ANK region, alternatively, that such mutation requires more than one amino acid substitution and therefore cannot be readily generated by point mutagenesis.

Surprisingly few of the mutations generated randomly were within the conserved core of the ANK repeats (shaded black or grey in Figure 1). To determine the extent to which the most conserved residues cores were contributing to Swi6 function, we performed site-directed mutagenesis of the core regions of each repeat, both singly and in combination. In each case the G-T-L core residues of the repeat were changed to alanines. We found that these mutants varied in function at 25 degrees, but all of these mutations conferred a temperature sensitive phenotype to Swi6. This indicates that the entire length of the ANK domain is critical for the stability of the Swi6 protein. Mutations in the first repeat had the least effect, then the second, third and the fourth increased in importance. The multiple mutants presented a more complex picture, in that many of the multiple mutants had more activity than the single fourth repeat mutant. The most surprising of these is the Swi6 mutant with all four repeats mutated. In this case, activity was much higher, about half that of wild type. This remains a mystery. Our working hypothesis is that the repeats are involved in some form of redundant negative control, such that mutation in a subset of the repeats doesn't disrupt it, but mutation of all four repeats does. This data can be found in Appendix 1, Table 2.

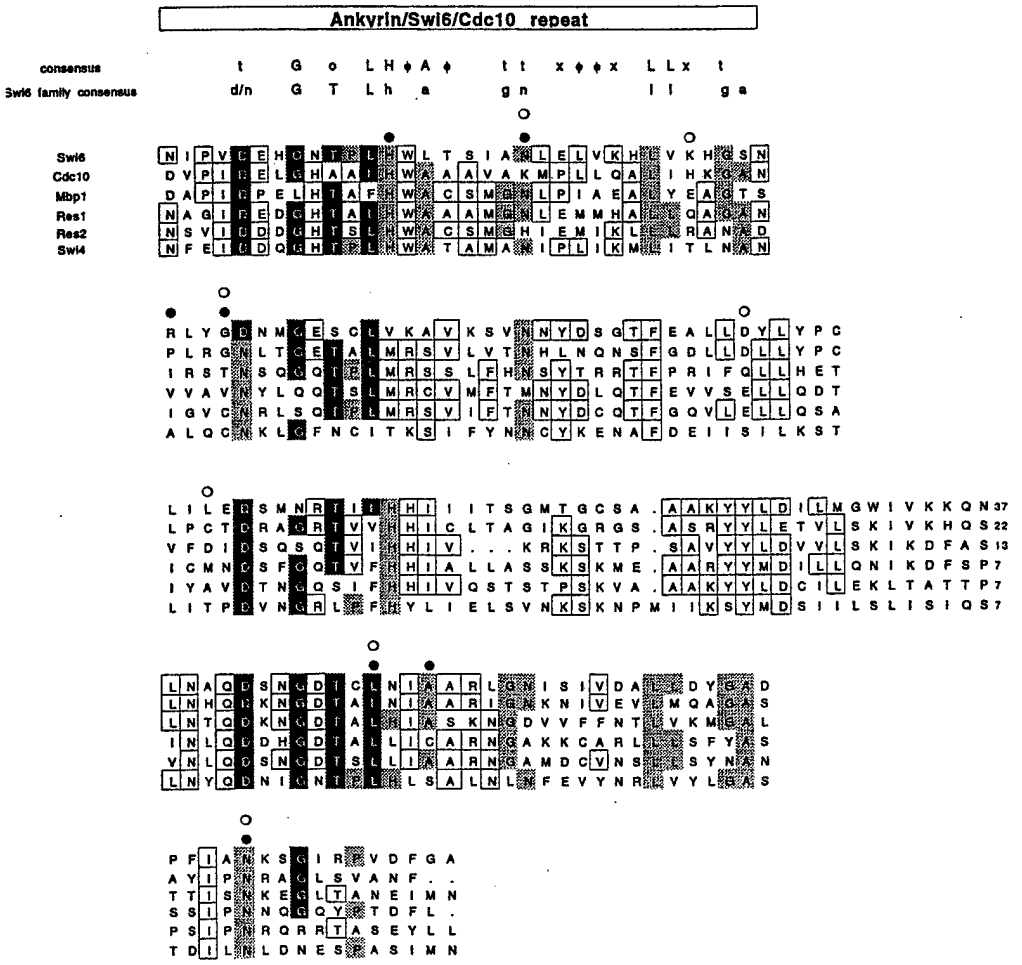

Figure 1. Alignment of the ankyrin repeat regions of a family of yeast transcription factors.

These three *S. cerevisiae* and three *S. pombe* transcription factors all contain a centrally located ankyrin repeat domain that consists of four full ankyrin repeats and vestiges of a fifth that are tandemly arranged. The black boxes are situated at the most highly conserved residues within the ankyrin motif, known as the "core" region. Each of the ankyrin repeats within the transcription factors has these regions conserved to some degree. The light grey boxes denote residues that are conserved across more than one repeat within this family and the white boxes highlight residues that are conserved within only one repeat of the ankyrin domain.

●: Single residue substitutions at these sites confer a temperature-sensitive phenotype to Swi6;

○: Mutant with residue substitutions at these locations were isolated more than once.

The recent publication of the crystal structure of a p53 binding protein, 53BP2, which contains four ankyrin repeats [11], has enabled us to estimate the positions of the ankyrin repeat mutations of Swi6 that we have generated. First, the four ankyrin repeats of Swi6 and 53BP2 were aligned with 24 other ankyrin repeat domains using CLUSTAL-W [12]. This multiple alignment provided a reliable means of aligning the two proteins' ankyrin repeats despite their low sequence identity (21%). Then, the coordinates for the 53BP2 structure, generously provided by N. P. Pavletich, were used in conjunction with the MODELLER program [13] to generate and refine a model of the Swi6 ankyrin domain. This model was strikingly similar to the 53BP2 structure. It was validated by three independent methods (PROCHECK. VERIFY-3D, and ERRAT (see figure 7 legend, Appendix 1) and all three indicated a high degree of reliability. The structure that 53BP2 adopts, and the one that the modeled Swi6 conforms to, resembles an "L" in profile, with continuous, tightly packed α-helices perpendicular to and connected by short β-hairpins. A ribbon diagram of the Swi6 ankyrin model structure is provided in Appendix 1, Figure 7. The locations of the core residues and five single-point mutants are labeled. The regularity of this repeat structure is apparent in this view, as is the conserved positioning of the core residues in each repeat.

Four ankyrin repeats occur in tandem within the yeast transcription factor family, and the four full repeats have been modeled on the basis of the structure of another protein which also contains four repeats. N500, one of the residues shown to be critical for Swi6 function in this analysis, is adjacent and C-terminal to the four-repeat structure. This asparagine, and the glycine that follows three residues downstream, are the residues that would be expected to begin a fifth repeat. However, the residues that follow do not conform to the consensus sequence. The significance of this partial fifth repeat is unclear, but the fact that it maintains appropriate spacing and sequence conservation within this family suggests that it may be important in the overall domain structure. This is corroborated by the fact that point mutations in N500 have been found which disrupt Swi6 function. In addition, there is another ankyrin repeat containing protein in yeast, Yar1, which also ends its ankyrin repeat sequence in the same manner [14].

Among the PCR-generated ankyrin repeat mutants, the most common identified mutation, isolated 10 times from three independent screens, was the glycine G347 position and all were substitutions of glycine for aspartate (D). G347, as pictured in Appendix 1, figure 7, lies on the back surface of the modeled structure, in the β-hairpin connecting ankyrin repeats 1 and 2. As such, changes at that position are unlikely to destabilize the structure itself. Rather, the loss of function that this mutation causes suggests that this residue may be part of a surface on which there is a critical interaction with another protein, or with another domain of the Swi6 protein. This is also likely to be the case for the gain of function mutation, N330K, which does not confer a conformational change to the modeled Swi6 ankyrin repeat.

The four other single mutations generated in this study that resulted in temperature-sensitive Swi6 proteins are all predicted to result in unfavorable interactions within the ankyrin repeat itself when modeled onto the structure. These findings help to validate the model and give us new insight into the key intramolecular interactions that occur in this structure. The core leucine (L) at position 474 changed to serine (S), and alanine (A) 477 changed to threonine (T) (Appendix 1, Figure 8A): both disrupt the tight hydrophobic packing that is predicted to occur within neighboring resides. In the case of histidine (H) 323 (Appendix 1, Figure 8B), which is replaced with arginine (R), the hydrogen bonds predicted to be formed by the histidine with aspartate (D) 348 are disrupted and the larger arginine side chain would be expected to perturb the local structure due to steric hindrance.

Finally, the space filling models depicted in panels A and B of Figure 9 (Appendix 1) clearly show the effect predicted by substitution of R344 for glycine (G). In the model, the loss of the bulky side chain of the arginine creates a cavity on the surface of the structure which exposes the hydrophobic side chains of L322 and L373. This cavity is large enough to make this region accessible to water and would be expected to destabilize the structure.

Subsequent to the publication of this work, the crystal structure of the Swi6 ANK domain was published [15]. These authors pointed out that their structure confirmed the structure we predicted with our model and the effects that these mutations would have.

TASK 3. Analyzing ANK repeat mutants for loss of specific functions *in vivo*.

Our screen for ANK repeat mutants was carried out with an *HO:lacZ* reporter group, which enabled us to assay Swi6 transcriptional activity with a simple colorimetric assay. We have since assayed these mutants using simplified promoter constructs. Specifically we have asked if these mutants show differential activity upon MCB versus SCB binding sites using specific reporter constructs which differ only in the DNA binding sites. Both sites are known to be bound by Swi6 [16-21], but it is unclear whether their binding would be affected similarly by these mutants. To obtain this data, we have compared lacZ levels produced from a set of reporter plasmids in yeast strains with different ts ANK mutations at nonpermissive temperature. The four reporter constructs we used had as a UAS (upstream activating sequences) in their promoters 1) a fragment of *HO* promoter, 2) a fragment of *TMP1* promoter, 3) a synthetic trimer of SCB elements and 4) a synthetic trimer of MCB elements (*TMP1* and *HO* fragments contained activating sequences that have MCB and SCB elements, respectively, in the native context). There was some variation between the different ts mutants as to the degree of defects in SCB versus MCB transcription, but all of them were defective at both types of promoter elements. Therefore, our results obtained so far suggest that the MCB and SCB transcription functions of Swi6 are not carried out by separate domains within the ANK repeat region.

To allow a direct comparison between SCB and MCB activation, we introduced a high-copy reporter construct carrying three tandem repeats of either MCB or SCB elements into the BY660 (*ho, swi6::TRP1-197*) strain carrying the mutant *swi6* allele on a low-copy plasmid. This enabled us to compare SCB and MCB activation within an equivalent context. Figure 6 (Appendix 1) shows that most of the PCR-derived and site-directed mutants cause a more severe MCB transcriptional defect when compared with SCB transcriptional activity. The most notable differences are seen with *swi6*-1234, which has 44% activity on SCBs and 0% on MCBs, *swi4*-124, which has 40% SCB activity and 0% MCB activity, and *swi6*-421, which has 48% of the wild-type SCB activity compared with 14% of the wild-type MCB activity. In addition, there are a number of mutants (*swi6*-1000, -200, -123, -405, -417, and –424) that show comparably decreased levels of both SCB and MCB activity when measured from these analogous promoter constructs. The fact that the vast majority of ankyrin repeat mutants are not equivalently defective with respect to MCB and SCB activation indicates there are differential effects upon the Swi4-Swi6 and Mbp1-Swi6 transcription complexes. This is somewhat surprising since the C termini, and not the ankyrin repeats of these proteins, are sufficient for the interaction between Swi6 and Swi4 [6, 22] and between Swi6 and Mbp1 [23]. Interestingly, all of the mutants are more defective in MCB activation than in SCB activation, despite the fact that they were selected for their inability to act at the *HO* promoter, which is activated by SCB elements. This suggests that the Swi6-Mbp1 interaction or activity has a stronger dependence either upon the ankyrin domain itself or upon other residues that are brought into proper position by the ankyrin domain structure.

We have also analyzed the Swi6 ANK mutants for two other phenotypes conferred by *swi6* deletion mutants: 1) the inability to recover from alpha factor [24], and 2) hydroxyurea sensitivity [25]. Interestingly, although the ANK mutants are highly defective in transcription, they appear to behave as the wild type in these two other assays. This is difficult to understand based upon current knowledge. It is possible that once global searches to identify all the Swi4/Swi6-dependent promoters are identified, candidate promoters that might be responsible for these phenotypes can be deduced and tested.

TASK 4. Biochemical characterization of the ANK repeat SWI6 mutants and potential ANK interacting proteins with regard to their DNA binding activities.

To test whether the inability of the Swi6 ankyrin mutant proteins to activate SCB transcription is due to a defect in the ability of Swi4-Swi6 complex to bind DNA, we compared the DNA binding activities of these mutant proteins to that of a wild-type Swi6 by band-shift analysis. Using whole cell extracts derived from the wild-type, *swi6-405*, and *swi6-406* strains and a fragment of the *HO* promoter as the SCB DNA probe, we find that the wild-type complex migrates predominantly as a single lower complex (Appendix 1, Figure 3A). Extracts from the *swi6-406* strain contain a complex that binds DNA about as efficiently as wild type, but the complex formed migrates more slowly (referred to as the upper complex). The *swi6-405* extract produces both upper and lower complexes in approximately equal amounts. These differences in mobility could be due to differences in the protein composition of the complexes or to modification or conformational changes within the Swi4-Swi6 mutant complexes. The variations in steady state levels of Swi6 in the temperature-sensitive mutants compared with that in the wild type could also influence the amount and type of complexes that form.

To eliminate some of these complications, we have used in vitro translated Swi4 and Swi6 in the DNA binding reactions. All of the ankyrin repeat mutant plasmids, as well as the wild-type *SWI6* plasmid, were transcribed and translated and found to yield comparable levels of Swi4 and Swi6 protein products (Appendix 1, Figure 3B and data not shown). DNA binding reactions using the in vitro translation products from *swi6-406* and wild-type *SWI6* plasmids were analyzed in parallel with those produced from whole cell extracts. The resulting band-shift patterns are qualitatively similar. Since the in vitro translated Swi4 and Swi6 were the only yeast proteins added to the reaction mixtures, it is unlikely that the tendency of the *swi6-406* ankyrin repeat mutant to form upper complexes is due to binding of additional proteins to the DNA-protein complex. It is also unlikely that differences in protein modification are responsible, since the proteins are translated in a rabbit reticulocyte lysate system. The possibility that proteins in this lysate could bind or modify the yeast protein-DNA complex cannot be excluded, but to produce these results, the protein has to be specific for the Swi6 mutant complexes. The protein would also need to be present in both rabbit and yeast cells, because the band shift is identical whether the mutant protein is translated in vitro or harvested from yeast.

To observe the extent of variation in DNA binding activity, the collection of temperature-sensitive ankyrin repeat mutants of Swi6 were translated in vitro and surveyed for DNA binding activity at two temperatures. Most of the Swi6 mutants retained the ability to complex with Swi4 and bind SCB elements at the nonpermissive temperature. Frequently, however, the mobility of the ankyrin mutant complex was noticeably altered (Figure 4, Appendix 1) and migrated as an upper complex that could not be distinguished from that of *swi6-406*. The wild-type (*SWI6+*) band-shift pattern shows predominant lower band at 25 °C and a more dispersed pattern, including both upper and lower

complexes, at 37 °C. From the band-shift assay, the *swi6* mutants can be grouped into four categories. The first group of mutants [*swi6-406, -407* (G347D), and *–401*] all form the upper complex predominantly at both the permissive (25 °C) and nonpermissive (37 °C) temperatures. The second group of mutants [*swi6-405* and *swi6-410* (H323R), *-422, -421, -424* (R344G), *-402*, and *–417* (A477T)] form the upper complex at the nonpermissive temperature and both the upper and lower complex at the permissive temperature. The third phenotype is overall reduced binding, even at the permissive temperature, seen with mutants *swi6-401, -417* and *–422*. The fourth group of mutants: *swi6-409* (N500T), *-415, -420, -423,* and *–418* (N500Y), show no binding defect at either temperature. Most of the mutants show altered binding characteristics with a propensity to form the upper complex, which is exacerbated by elevated temperatures. A minority have reduced overall DNA binding. Formation of the upper complex therefore correlates with the loss of transcriptional activation, which is also enhanced at high temperatures. Despite the considerable variability in the DNA binding complexes formed, the upper complex that most of the mutants display migrates at a very similar position in the band-shift gel. Since this is not likely to be due to changes in protein composition or modification states, we speculate that it may be the result of a global change in the conformational state of the Swi4-Swi6 complex which these mutations induce to varying degrees.

To measure the DNA binding defect associated with the core substitution mutants, we employed in vitro translation and band-shift assays as before, incubating the reaction mixtures at both 25 and 37 °C. As stated above, the wild-type Swi4-Swi6 complex exist as a combination of upper and lower complexes on SCB DNA, with a predominant lower complex at 25 °C. The core mutants all form the upper complex exclusively at both temperatures (Figure 5 of Appendix 1 shows the 25 °C experiment). The ability to form the upper band-shift complex did not localize to mutations within a particular core region. Thus, even more so than with the PCR-generated repeat mutants, we see a dramatic but uniform shift in mobility of the DNA binding complex with the core mutants of Swi6. This, and the temperature sensitivity of all these mutants, suggests that the conserved core residues of all four repeats are important for maintaining the native structure of Swi6 and/or Swi4-Swi6 complex.

TASK5. A screen for high copy suppressors of ANK ts mutations.

The two of temperature sensitive ANK mutants of *SWI6* genes were used to identify high copy suppressors. These mutants were transformed with a 2μm-based yeast genomic library [26] and about 60,000 transformants were obtained for each. Detailed methods are available in Appendix 2. We recovered a total of 8 suppressor plasmids from *swi6-406* and 19 from *swi6-405* transformants. These are listed in Table 1, Appendix 2. Several suppressors were not pursued further because they activated *ho::lacZ* expression equally strongly in *swi6-405* and *swi6Δ* cells and thus were completely independent of Swi6. Most of the suppressors suppressed the *ho::lacZ* expression defect to some extent in the absence of Swi6, but only one (c19) could suppress in the absence of Swi4. This requirement for Swi4 suggests that the majority of suppressors enhance Swi4-mediated activation, rather than by causing a general derepression of transcription. c15 showed no suppression of the *ho::lacZ* transcription defect in *swi4* or *swi6* deletion strains and thus was the best candidate for an allele-specific suppressor.

We obtained some sequence information for all of the suppressors and have searched genomic databases with these sequences to obtain full maps of the DNA inserts.

In each case more than one open reading frame was present on the insert. We have subcloned insert fragments to determine which of the open reading frames were encoding suppressors. The results of mapping and subcloning showed that previously cloned genes, *MSN1* and *NHP6A* were responsible for suppression phenotype of several of the clones. c23 appears to carry a different fragment of chromosome XV and c2 carries a fragment from chromosome XIII. The open reading frames present on these fragments have not been characterized previously. We have determined which open reading frame of c2 is responsible for suppression. It is a new gene of unknown function (ORF YM8520.13c) that we called *PIB1* (Partially Independent Bypasser of Swi6). *PIB1* has no close homologues in yeast or in higher eukaryotes, however it resembles several transcription factors. Interestingly, it bears some structural similarity to a *Drosophila* transcription factor Dorsal, which is known to interact with the ANK repeat containing regulator Cactus [27]. We have disrupted *PIB1* and found that it is not essential for yeast viability. Double mutants with disruption of *PIB1* and either *SWI4* or *SWI6* are also viable. Also the steady state HO levels are unchanged in the *pib1Δ*, so this gene has not been pursued further.

Previously identified genes, *MSN1* and *NHP6A,* were responsible for suppression phenotype of c4 and c15, respectively. *MSN1* was originally cloned as a high copy suppressor of a temperature sensitive *SNF1* kinase mutant for its ability to restore *SUC2* expression [28]. It acts as a transcriptional activator when fused to LexA and does not have any specific DNA binding activity [28]. *NHP6A* has also been identified previously [29] and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of bending DNA [30]. RNA analysis showed that both *MSN1* and *NHP6A* exert their function at the mRNA level, rather than by affecting β galactosidase stability or activity.

To see if *MSN1* and *NHP6A* are normally involved in the transcription of the Swi4/Swi6-regulated promoters, we isolated mRNA from exponentially growing cultures of strains with or without *MSN1*, or *NHP6A* and *B* gene products and compared the levels of *HO* and *CLN1* transcripts in these strains to the wild type strain by S1 protection (Figure 2, Appendix 2). The *msn1Δ* strain expresses about three to five-fold less *HO* transcript and the *nhp6abΔ* strain shows a two-fold drop in *HO* transcript compared to wild type. Interestingly, there is little or no effect of *msn1Δ* on another Swi4/Swi6-regulated promoter, *CLN1* (data not shown) but the *nhp6abΔ* has a similar two-fold effect on *CLN1* all methods are described in Appendix 2.

To see whether there is a direct interaction between Nhp6A and Swi6, we immunoprecipitated Swi4 or Swi6 proteins out of wild type extracts carrying HA-Nhp6A and then immunoblotted with anti HA antibodies to detect HA-Nhp6A. Despite the fact that this HA-tagged Nhp6A is functional and can suppress the *swi6-405* transcription defect (Figure 1b, Appendix 2), there was no indication that HA-Nhp6A coprecipitates either with Swi4 or Swi6 under the same conditions that we use to detect Swi4/Swi6 association [6].

We then prepared fusions of Nhp6A and Msn1 proteins with Gst. The *GST-NHP6A* and *GST-MSN1* fusions were put under the control of *GAL1-10* promoter and expressed in a wild type strain. Because of their toxicity when overproduced, we purified these Gst fusions from cells grown in raffinose and then induced by galactose addition for only 3-4 hours. Though we could purify the fusion proteins from these cells under low stringency conditions, there was no detectable Swi6 copurifying with either of them. We also purified these Gst fusion proteins from *E. coli* on glutathione beads and then incubated the fusion-bound beads with recombinant Swi6 [6], or *in vitro* translated Swi4/Swi6 complex [31]. Even under these conditions, we could not detect interaction between Swi4, or Swi6, and the fusion proteins.

MSN1 has been originally cloned as a high copy suppressor of a temperature sensitive *SNF1* kinase mutant [32]. It has activator properties and has an extremely weak

and nonspecific DNA binding activity. *MSN1* has been also selected by other groups as *FUP1*[11] and *PHD2*, an activator of pseudohyphal growth [6] as well as a protein capable of activating mating specific genes when overproduced [33]. Therefore, it is likely that *MSN1* is a non-DNA binding transcription activator, or coactivator, that is utilized by several unrelated promoters. Strains deleted for *MSN1* grow slower than the wild type suggesting that *MSN1* is involved in the regulation of genes important for viability. We found that in *msn1* deletion strain the transcription of *HO* is reduced to 20-30% of the wild type. Thus, *MSN1* is required for maximal expression of *HO*.

NHP6A has also been identified before [34, #238] and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of looping DNA [20]. *NHP6A* has a close homologue in yeast, *NHP6B*, which encodes a similar protein with potentially overlapping activity [34]. Strains with deletions in both *NHP6A* and *NHP6B* have multiple phenotypes, including dramatically reduced growth rates. Both NHP6 proteins have long been implicated in facilitating gene expression by inducing favorable chromatin conformations on the promoters, however, this is some of the first direct evidence that *nhp6A*-6B deletion strains impair transcription.

KEY RESEARCH ACCOMPLISHMENTS

- **Some portion of the ANK domain of Swi6 is accessible to antibodies in the native state.** Swi6 is an antigenic protein, as are the ANK repeats in isolation. The fact that at least one monoclonal antibody generated against the ANK domain can be used to immunoprecipitate Swi6 from yeast extracts suggests that some portion of the ANK domain is accessible in the native state. This was corroborated by our modeling studies and verified in the crystal structure of Swi6.

- **There are at least four functional ANK repeats in Swi6.** The fourth repeat of Swi6 is the most critical for Swi6 function, but the conserved core of each repeat is important for Swi6-mediated activation of HO:lacZ transcription.

- **Many residues within the ANK domain are critical for function.** Mutants across the spectrum of severity have been isolated from this screen. We have focussed upon temperature sensitive ones, but many non-conditional null mutants have also been isolated. It is clear that the ANK domain of Swi6 contains a large number of residues that are critical for its function.

- **Mutations that cause loss of Swi6 function at high temperature are found throughout the ANK domain**. There is some clustering of mutations at each end of the ANK domain, but each ANK repeat is important. The only region lacking mutations is the non-structured part of the spacer.

- **We have identified some of the critical residues: R344, G347, D375, A477 and N500, whose substitution is enough to compromise the activity of the whole region.** All but one of these (G347) compromise the structural integrity of ANK domain.

- G347 is a surface residue critical for Swi6 function. This mutation also causes a dramatic conformation change in the Swi4/Swi6 complex, so we expect that **G347 resides within a domain of contact between Swi4 and Swi6, or an intramolecular interface.**

- Most if not all of the mutants that we looked at were defective both in SCB- and MCB-driven transcription, indicating that **the two activities are not separable by mutation in the ANK domain.**

- **Hyperactivating mutations cannot occur in ANK region**, or such a phenotype requires more than a couple of amino acid substitutions and therefore cannot be generated by this mutagenesis procedure.

- Site-directed mutations within the conserved cores of the ANK repeats all confer a temperature sensitive phenotype to Swi6. **This indicates that the entire length of the ANK domain is critical for the stability of the Swi6 protein.** Mutations in the first repeat had the least effect, then the second, third and the fourth increased in importance.

- The enhanced activity of multiple repeat mutants suggest the possibility that the repeats are critical for some form of negative control over Swi6 activity.

- **We have identified three genes that can suppress ts ANK defects of SWI6.** *PIB1* is a new gene, with homology to some other transcription factors, but no known function. The other two: *NHP6A* and *MSN1*, are previously identified genes. We have established that two of these genes are required for maximal *HO* expression. **NHP6A facilitates Swi4/Swi6 complex formation on DNA,** but we have no evidence of direct interaction between Swi6 and any of these proteins. Thus, **we conclude that they act indirectly to facilitate Swi6-dependent transcription.**

- Most of the ANK mutants show a coordinated loss of both SCB and MCB-activation. However, there is a more severe defect in MCB transcriptional activity than in SCB transcriptional activity in several mutants. **This indicates that these two activities are not carried out by different repeats within the ANK domain, and that MCB activity is more dependent upon the structural integrity of the Swi6 protein.**

- There is a surprising range of effects of these ANK mutants upon DNA binding. Some mutants are extremely defective in DNA binding. Others show a predominant upper complex compared to the wild type cells. Since these assays are carried out with only Swi4 and Swi6 proteins produced from reticulocyte lysates, it is unlikely that the reduced mobility of these upper complexes is due to the addition of another protein in the complex. Rather, we conclude that it is most likely that the **ANK mutations are causing a fairly radical change in the conformation of the complex.**

REPORTABLE OUTCOMES

Bibliography of manuscripts and meeting abstracts

Ewaskow, S. P., J. M. Sidorova, J. Hendle, J. E. Emery, D. Lycan, K. Zhang, L. Breeden. (1998) Mutation and modeling analysis of the Saccharomyces cerevisiae Swi6 ankyrin Repeats. **Biochemistry** 37:4437-4450.

Sidorova, J.M. and L. Breeden (1999) The MSN1 and NHP6A genes suppress SWI6 defects in Saccharomyces cerevisiae. **Genetics** 151:45-55.

Lycan, D. E. B. Bollinger, K. Stafford, L. Breeden. Isolation of a novel ankyrin-repeat containing gene from S. cerevisiae. American Society for Cell Biology 1994, San Francisco, CA.

Lycan, D.E., J.C. Emery, S. Ewaskow, J. Sidorova, L. Breeden Point mutations in the ankyrin domain of Swi6 that affect SCB function. American Society for Cell Biology 1995, San Francisco, CA.

Breeden, L., J. Partridge, J. Sidorova, C. McInerny, and G. Mikesell Transcriptional control of the early events in the budding yeast cell cycle. ICN-UCLA symposium 1996, Taos, New Mexico

Ewaskow, S., J. Sidorova, J. Hendle, J. Emery, D. Lycan, K. Zhang, L. Breeden Mutation and modeling analysis of the S. cerevisiae Swi6 ankyrin repeats. 1997 US Dept. of Defense Breast Cancer Meeting, Washington D.C.

Cell lines (See Table 1 in Final Report)

20 monoclonal cell lines derived from injection of the Swi6 ANK domain

3 monoclonal cell lines derived from injection of the full length Swi6 protein

Employment and Research opportunities

Dr. Sandra Ewaskow was awarded a 1 year fellowship from the College of American Pathologists Foundation (1995).

Dr. Sandra Ewaskow was awarded a five year Physician Scientist Award (K11) from the National Institutes of Health for 12/95 to 1/00

Dr. Julia Sidorova was awarded a three year Leukemia Society of America Senior Fellowship for 7/99 to 6/02

CONCLUSION

Within the four ankyrin repeats in this family of transcription factors, three levels of conservation are observed. There are "core" residues, which are identical, as well as residues with chemical similarities that are conserved throughout all four of the repeats. Second, there are specific residues which are shared by repeats 1 and 4, making them much more similar to each other than the other repeats in these or any other set of ankyrin repeat proteins. Third, there are residues which are conserved within the individual repeats of all of the family members, but which differ widely between repeats. This study provides evidence that the core residues of all four of the repeats are important, but whether this is due to a structural requirement for a four-repeat domain or whether these repeats actually have different roles is unclear. The fact that the mutations in the core residues all result in temperature sensitivity and all cause what appears to be a dramatic conformational change in the Swi4-Swi6 DNA binding complex is evidence that the role of these residues may be structural.

Modeling studies of our single-point mutants also show that all but one of these mutations are likely to disrupt the structure of the ankyrin repeat domain. The one exception is G347D, which may define a surface on which there is a critical interaction. This interaction is most likely to be between Swi6 and Swi4, or with another part of Swi6, as this mutant also causes a dramatic shift in the ternary complex between Swi4, Swi6, and DNA on band-shift gels. Our transcript measurements show that all of the ankyrin repeat mutants, which were initially selected for defects in SCB-driven transcription in the context of the native *HO* promoter, have an even more severe defect in MCB-driven transcription. This greater dependence upon the ankyrin repeat domain for Mbp1-Swi6 than that for Swi4-Swi6-dependent transcription indicates differences in the contacts within the two complexes. These residues may be identifiable with more exhaustive genetic screens.

Ankyrin repeats were initially identified as statistically significant homologies between the repeats of Swi6 and its nearest *S. pombe* relative, Cdc10. Ankyrin repeats were also found in the Notch protein of *Drosophila* and the *Caenorhabditis elegans* lin-12 protein, both of which are also highly related [5]. Since then, ankyrin repeats have been identified in more than 100 proteins with highly diverse functions [35]. The sequence consensus has been relaxed considerably over that time, and the discovery of new members of the family is aided as much by the fact that they are nearly always present as tandem repeats as by their sequence similarities.

The function of the ankyrin repeat has been investigated in many different systems, and several key protein-protein interactions have been shown to depend on them [36-40]. However, the repeats do not appear to interact with each other, nor has any other "signature" sequence been identified that is diagnostic of an ankyrin repeat-interacting protein. This, coupled with the fact that the proteins in which these ankyrin repeats are found have highly diverse functions and are located in a myriad of different cellular compartments (spider venoms, membrane transport proteins, and transcription factors), suggests that these repeats do not have a common function or binding partner. Rather, they should be viewed as structural units, which confer a particular type of protein fold. Gorina and Paveletich [11] have noted that ankyrin repeats form a novel L-shaped structure. Our study provides evidence that the conserved residues within each repeat are required to produce this structure, and as such, they provide a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues, which are unique to the different classes of ankyrin repeat-containing proteins, are responsible for specific interactions with other proteins and for providing the biological specificity and function to

the ankyrin repeat proteins. The conserved residues, which are the defining feature of an ankyrin repeat, may play a strictly conformational function. Thus, the ankyrin repeat may be more appropriately viewed as a novel type of protein fold which provides a stable structure with surfaces that can be tailored for many different macromolecular interactions. Our data indicate that the ankyrin repeats of Swi6 are critical for the thermostability of Swi6 and for maintaining the proper conformation of the ternary complex between Swi4, Swi6, and DNA. It is still possible that the unique faces of the Swi6 ankyrin repeat domain form a binding site for another protein that has not yet been identified. However, it is more likely that these repeats provide a rigid structure that holds the Swi4-Swi6 complex in a precise and functional spatial arrangement with respect to the DNA.

ANK repeats were first found in the Swi6 transcription factor of *Saccharomyces cerevisiae* and since then were identified in many proteins of eukaryotes and prokaryotes. These repeats are thought to serve as protein association domains. In Swi6, ANK repeats affect DNA binding of both the Swi4/Swi6 and Mbp1/Swi6 complexes. We have described generation of random mutations within the ANK repeats of Swi6 that render the protein temperature sensitive in its ability to activate *HO* transcription. Two of these *SWI6* mutants were used in a screen for high copy suppressors of this phenotype. We found that *MSN1*, which encodes a transcriptional activator, and *NHP6A*, which encodes an HMG-like protein, are able to suppress defective Swi6 function. Both of these gene products are involved in *HO* transcription, and Nhp6A may also be involved in *CLN1* transcription. Moreover, since overexpression of *NHP6A* can suppress caffeine sensitivity of one of the *SWI6* ANK mutants, *swi6-405*, other *SWI6*-dependent genes may also be affected by Nhp6A. We hypothesize that Nhp6A and Msn1 modulate Swi6-dependent gene transcription indirectly, through effects on chromatin structure or other transcription factors, since we have not been able to demonstrate that either Msn1 or Nhp6A interact with the Swi4/Swi6 complex.

SO WHAT?

At the time that this research was proposed there was a real possibility and, indeed, an expectation that ANK repeats carried out a common function, that probably had to do with protein-protein interaction. These biases were guided by the discoveries of other short repeated motifs that, like SH2 domains, that were recognizable by their "signature" sequence and conferred very specific binding to another conserved class of proteins. It was our hope that if the same was true with ANK repeats, we could use the simple genetic system of yeast to identify the class of binding partner that ANK repeats associated with. Since a considerable number of defects in ANK repeat proteins had been correlated with tumor development, it was apparent that understanding the role of these repeats in molecular detail could provide new insight into oncogenesis. It was on this basis that we began this research.

Our results have shown that the conserved residues within the ANK repeat play a critical structural role in Swi6. This work and the results with other ANK repeat proteins compel a different view of these repeats, as structural units that confer a particular type of protein fold. Now, it is clear from the Xray structures of four different types of ANK repeat proteins that their overall structure is very similar. Each of these proteins interacts with other proteins, but different surfaces of the ANK domain are engaged in these interactions. Thus, the ANK repeats represent one way to fold a polypeptide chain into a stable structure, and this structure provides a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues within the repeats are responsible for the specific protein-protein interactions of each class of ANK repeat proteins.

This new view leads me to reject my original hypothesis, that understanding the function of the Swi6 ANK repeats would lead to insight into the function of oncogenic

ANK repeat proteins. More importantly, perhaps, it also suggests that the right path to their understanding is to focus upon the nonconserved, surface residues within these oncogenic repeat proteins and search for proteins which interact with them.

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APPENDICES

APPENDIX 1

Ewaskow, S. P., J. M. Sidorova, J. Hendle, J. E. Emery, D. Lycan, K. Zhang, L. Breeden. (l998) Mutation and modeling analysis of the Saccharomyces cerevisiae Swi6 ankyrin Repeats. **Biochemistry** 37:4437-4450.

APPENDIX 2

Sidorova, J.M. and L. Breeden (l999) The MSN1 and NHP6A genes suppress SWI6 defects in Saccharomyces cerevisiae. **Genetics** 151:45-55.

Mutation and Modeling Analysis of the *Saccharomyces cerevisiae* Swi6 Ankyrin Repeats

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Mutation and Modeling Analysis of the *Saccharomyces cerevisiae* Swi6 Ankyrin Repeats†

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ABSTRACT: The Swi4−Swi6 family of transcription factors confers G1/S specific transcription in budding and fission yeast. These proteins contain four ankyrin repeats, which are present in a large number of functionally diverse proteins and have been shown to be important for protein−protein interaction. However, no specific sequence has been identified that is diagnostic of an ankyrin repeat-interacting protein. To determine the function of the ankyrin repeats of Swi6, we generated both random and site-directed mutations within the ankyrin repeat domain of Swi6 and assayed the transcriptional function of these mutant *swi6* alleles. We found six single mutations, scattered within the first and the fourth repeats, that generate a temperature-sensitive Swi6 protein. In addition, we found that alanine substitutions for the most conserved residues in each repeat were highly deleterious and also confer temperature sensitivity. Most of these *swi6* alleles are able to form ternary complexes with Swi4 and DNA, but these complexes display reduced mobility in band-shift gels, suggesting a dramatic conformational change. We have modeled the ankyrin repeats of Swi6 using the coordinates derived for 53BP2 and find that, despite its low level of sequence conservation, these modeling studies and our mutation data are consistent with Swi6 having a structure very similar to that of 53BP2. Moreover, all but one of our single mutants and all of the site-directed mutants disrupt critical structural features of the predicted folding pattern of these repeats. We conclude that the ankyrin repeats play a major structural role in Swi6. Ankyrin repeats are unlikely to have inherent protein or DNA binding properties. However, they form a characteristic and stable structure with surfaces that may be tailored for many different macromolecular interactions.

The ankyrin−Swi6−Cdc10 repeat was first identified in a family of yeast transcription factors (*1*, *2*). Included within this family are the *Schizosaccharomyces pombe* transcription factors Cdc10, Res1, and Res2 and the *Saccharomyces cerevisiae* transcription factors Swi6, Swi4, and Mbp1 (Figure 1). Members of these groups associate with one another through their C termini (*3*, *4*). The target genes for these transcription factors are expressed at the G1/S transition in yeast and include DNA synthesis genes (*5*, *6*), *HO* endonuclease (*7*), and the G1 cyclins (*8*, *9*) that are required for progression through the yeast mitotic cell cycle. Swi6 is one of the transcription factors that regulates gene expression during G1/S, either as a complex with Mbp1, with which it binds MCB [*Mlu*I cell cycle box (ACGCGTNA)] DNA elements (*5*, *6*, *10*), or in combination with Swi4, with which it binds SCB [*SWI4/6*-dependent cell cycle box (CACGAAAA)] elements (*11*−*13*) as well as MCB-like elements (*14*). Swi6 is not known to bind DNA directly; rather, both Mbp1 and Swi4 confer the DNA binding ability to the complex through their N termini.

A common central motif consisting of four ankyrin repeats is present among all the known G1/S specific yeast transcription factors. Ankyrin repeats typically occur as four or more continuous copies of a 33-amino acid sequence characterized by the consensus sequence ----t--G-o-LHϕAϕ--tt-x$\phi\phi$x-LLx-t-- (Figure 1), where t indicates a residue frequently found in turns, x a polar residue, ϕ a hydrophobic residue, o a serine or threonine residue, and capital letters indicate highly conserved amino acids (*15*). A further defining feature of the yeast transcription factor ankyrin repeats is the central core region, denoted by the consensus sequence G-T-L (Figure 1). Ankyrin repeats are present in a large number of functionally diverse proteins and have traditionally been considered sites of protein−protein interaction. Circular dichroism and NMR studies of the tumor suppressor protein $p16^{INK4A}$, which consists of four ankyrin repeats, have confirmed the predominantly α-helical nature of the ankyrin repeat region (*16*). The crystal structure of the p53 core domain bound to 53BP2 reveals that a single ankyrin repeat structurally consists of one β-hairpin and two α-helices (*17*).

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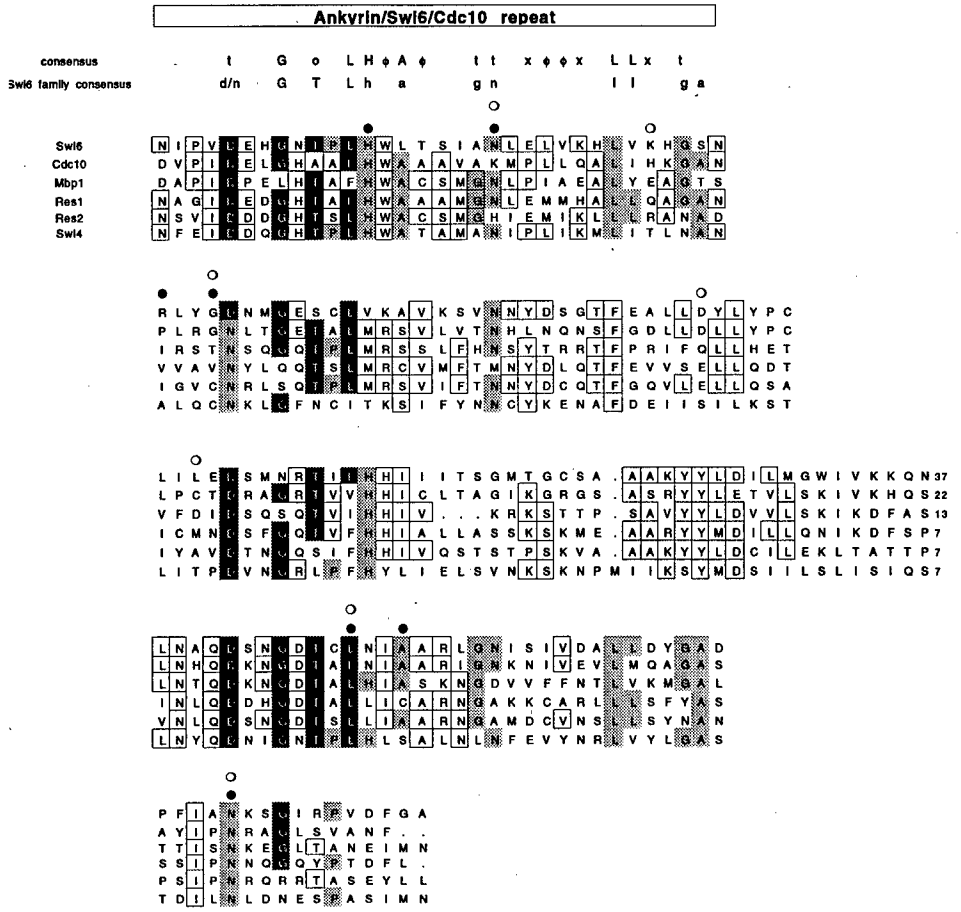


FIGURE 1: Alignment of the ankyrin repeat regions of a family of yeast transcription factors. These three *S. cerevisiae* and three *S. pombe* transcription factors all contain a centrally located ankyrin repeat domain that consists of four full ankyrin repeats and vestiges of a fifth that are tandemly arranged. The black boxes are situated at the most highly conserved residues within the ankyrin motif, known as the "core" region. Each of the ankyrin repeats within the transcription factors has these regions conserved to some degree. The light gray boxes denote residues that are conserved across more than one repeat within this family, and the white boxes highlight residues that are conserved within only one repeat of the ankyrin domain. (●) Single residue substitutions at these sites confer a temperature-sensitive phenotype to Swi6. (○) Mutants with residue substitutions at these locations were isolated more than once. x depicts a polar residue. ϕ depicts a hydrophobic residue. t depicts a residue found in turns.

Ankyrin repeats play an important role in Swi6, Swi4, and Cdc10 function. In Swi6, alanine substitutions of the first and fourth ankyrin repeat core residues result in decreased SCB binding and loss of transcriptional activation by the Swi4−Swi6 complex (*3*). In *cdc10*, the *S. pombe* homologue of *SWI6*, a group of conditional alleles were sequenced and the mutations clustered within the ankyrin repeat domain (*18*). Many of these single mutations mapped to the highly conserved ankyrin repeat core residues of the first and fourth ankyrin repeats. The ankyrin repeat domain of Swi4 is known to interact with the Clb2−Cdc28 kinase (*19*), and this has been postulated to inhibit Swi4−Swi6 transcriptional activity at SCB elements during the G2/M phase of the cell cycle.

The original alignment of Swi6 with other ankyrin repeat-containing proteins showed two repeats with high homology to the *Notch* and *lin-12* repeats, and these were used to define this 33-amino acid repeat (*1*). However, the identification of many more proteins containing ankyrin repeats, and the alignment of the five other members of the Swi4−Swi6 family of transcription factors, makes it clear that ankyrin repeats can be highly degenerate tandemly arranged sequences, and the Swi6 family may contain four full repeats followed by the N-terminal half of a fifth repeat (Figure 1) (*20*).

To determine if all of the Swi6 ankyrin repeats are important for Swi6 transcriptional activation, we used error-prone PCR to mutagenize the ankyrin domain of Swi6. Mutant plasmids were then tested for the ability of altered Swi6 proteins to activate SCB-regulated transcription. We obtained six single mutants and many mutants with combinations of double or triple amino acid residue substitutions that inactivate Swi6 as measured by the blue-white plate assay (*21*) for transcription of the *HO* promoter at permissive (25 °C) and nonpermissive (37 °C) temperatures. In addition, we made site-directed mutations of the most highly conserved residues within the core regions of each of the repeats. These also result in temperature sensitivity and confer band-shift mobility patterns that suggest an important structural role for each of the four repeats. To better understand the structural consequences of these ankyrin repeat mutations, we modeled the Swi6 ankyrin repeat region, using the coordinates obtained from the crystal structure of the four ankyrin repeats of 53BP2 (*17*), and found that Swi6 can adopt a very similar structure. The alanine substitutions in the conserved core leucines are likely to disrupt the regular secondary structure of the ankyrin repeat domain. Five of the six point mutants examined also disrupt the modeled structure, and the sixth mutant resides on the surface of the protein and may define a site of interaction.

EXPERIMENTAL PROCEDURES

PCR Mutagenesis of the Swi6 Ankyrin Repeats. Oligonucleotide primers BL58 (2.5 μM) (5′CCTGTAGATGAGCATGG3′) and BL202 (2.5 μM) (5′CCAAAATCCACGGGTCT3′) were used to perform mutagenic PCR in the presence of 5.0 mM deoxynucleotide triphosphate and either 0.5 mM (screen 1), 0.25 mM (screen 2), or 0.1 mM (screen 2) manganese chloride to facilitate nucleotide misincorporation (*22*). A *SWI6 CEN URA3*+ plasmid pBD1378 (0.05 μM) (*23*) was the double-stranded DNA template. To produce single-stranded templates from the double-stranded mutagenized PCR product, 25 rounds of PCR were performed using kinased primer BL202 (2.5 μM) and 1 μL of the double-stranded mutagenized PCR library product in a 50 μL reaction volume. The resulting single-stranded mutagenized product was then annealed to a single-stranded *SWI6* DNA template produced in the *dut− ung− Escherichia coli* strain (*24*). The heteroduplex plasmids were then introduced into a *dut+ ung+ E. coli* strain to propagate, generating the high (0.5 mM $MnCl_2$), middle (0.25 mM $MnCl_2$), and least (0.1 mM $MnCl_2$) mutagenized libraries. We isolated the mutagenized plasmids from *E. coli* using the alkaline plasmid prep procedure (*25*) and then introduced the plasmids into the *swi6* deletion strain BY600 (*MATa, ho:lacZ, swi6::TRP1−197, ura3, leu2−3,112, ade2, trp1, his3, trp1−1, can1−100, met*) using the lithium acetate method of transformation (*26*) and isolated those mutants that grew on uracil-deficient agar. Strains bearing the mutant *swi6* alleles were constructed by targeted integration (*27*) of the BY600 strain with linearized pRS305 plasmids carrying the *Hin*dIII−*Sma*I fragments of *swi6*. Both rich (YEPD) and minimal, selective (YC) growth conditions were as described previously (*28*).

Generation of Site-Directed Mutants. Mutant plasmids pBD1819 and pBD1822 were generated from a *Sac*I digest of pBD1028 and pBD1029, respectively, followed by ligation of the 1.5 kilobase fragment into the *Sac*I-digested pRS316. The remainder of the site-directed mutants were generated through substitution of the indicated core residues using oligonucleotide-directed mutagenesis of a single-stranded *SWI6* template (pBD1378) (*24*). The first ankyrin core region was mutated using primer Bd1035 (mutant base pairs are underlined) (5′GCTATTGAAGTTAACCAATGTGCTGGTGCGTTTGCGTGCTCATCTAC3′), the second using primer BL86 (5′GCTTTCACTGCGCATGCCTCCGCCATATTATCACC3′), the third using primer BL87 (5′GATATGGTGCGCAATTGCTCTAGCCATTGAGTCTTC3′), and the fourth using primer Bd1036 (5′GCTGCAATGTTAGCGCAAGCATCGGCATTCGAGTCCTGC3′).

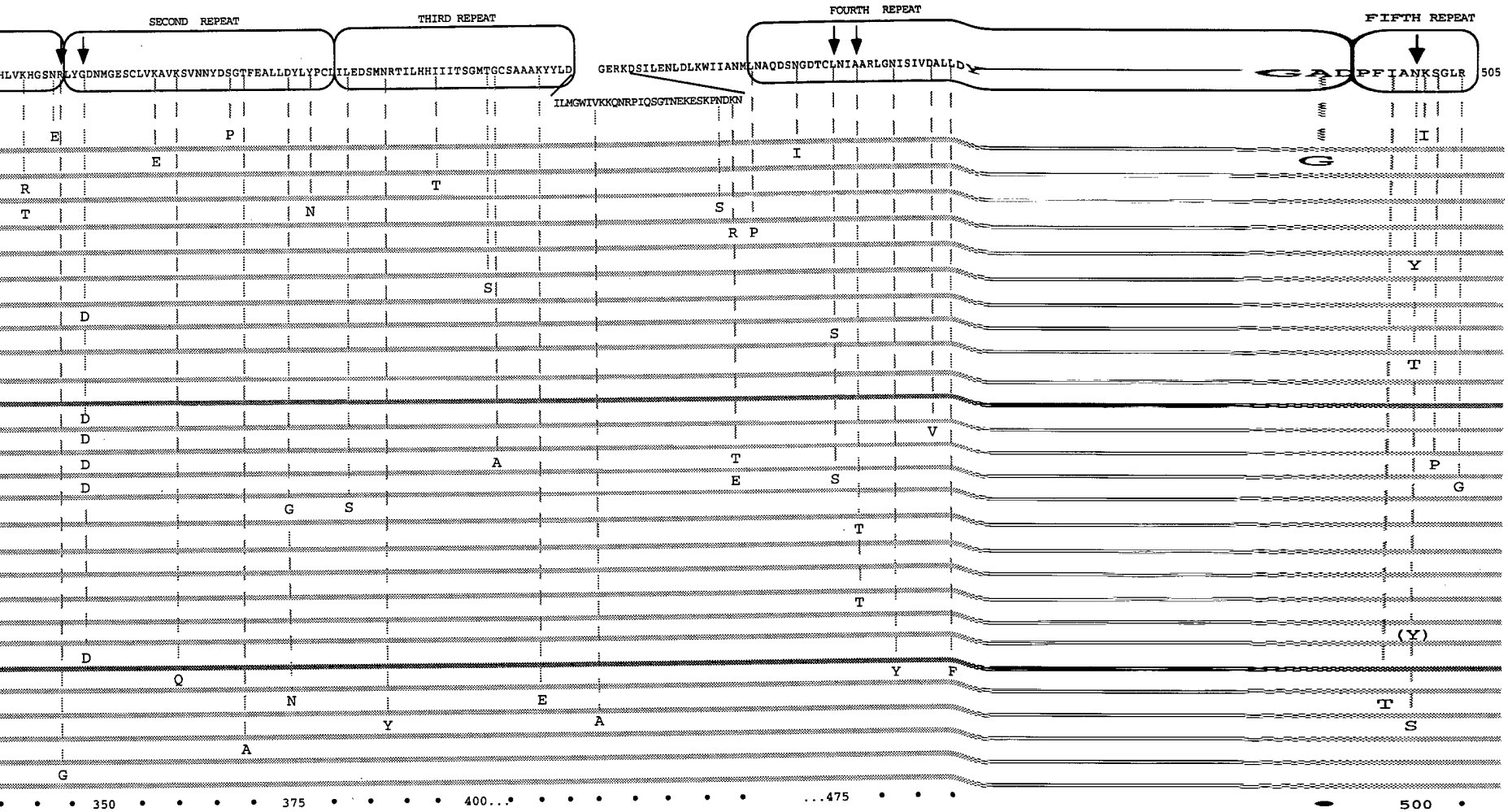
Identification of the Temperature-Sensitive Mutant Plasmids. BY600 (*swi6::TRP1−197, ho:lacZ*) was transformed with the PCR-generated libraries and grown on YC-ura plates at 25 °C, then replica plated, and grown at both 25 and 37 °C. The colonies were transferred to nitrocellulose filters and assayed for β-galactosidase activity as described (*21*). The *ho:lacZ* reporter construct for the blue-white screen has been described previously (*21*). Transformants that were blue at 25 °C and white at 37 °C were selected, as well as cells bearing null alleles, which were white at both 25 and 37 °C. Mutant plasmids were then isolated from the yeast strain and retransformed into the BY600 (*swi6::TRP1−197*) strain to confirm the dependence of the temperature-sensitive blue-white phenotype on the plasmid-borne *swi6* gene.

To identify mutant plasmids that had significant defects in Swi6 protein stability, extracts were prepared from the temperature-sensitive *swi6* mutants after incubation at 37 °C for 10−12 h. The differences in Swi6 levels are pronounced in some cases and could be contributing to the defective phenotype of ankyrin repeat mutants. Thus, the temperature-sensitive mutants that were selected for further characterization were those which maintain near wild-type levels of Swi6 protein and are likely defective due to a loss of function at high temperatures.

Generation of Yeast Strains. Yeast strains BY2214 through BY2223 were generated by ligating the 2.8 kilobase *Hin*dIII- and *Sma*I-digested *swi6* fragment from the mutagenized *URA*+ pRS316 construct into the *LEU*+ pRS305 vector (*29*). The pRS305 vector was then linearized by digesting with *Hpa*I (or *Nar*I for BY2220, -2214, -2215, -2216, and -2217) and transplaced into the chromosomal *leu2* locus of the BY600 (*swi6*Δ) strain using targeted integration as described previously (*27*). Integrated mutants were placed under *LEU* selection, and transplacement was confirmed with both PCR and sequencing.

Immunoblotting. Yeast cell cultures were grown overnight at 30 °C in selective media to an OD_{660} of <0.5, then diluted to an OD_{660} of 0.2, and split and grown at either 25 or 37 °C for up to 10−12 h (OD_{660} of 0.4−0.5). Protein extracts were made by lysing the cells with glass beads under hypotonic conditions in extract buffer [100 mM Tris (pH 8.0), 20% glycerol, 0.1% Triton X-100, and 1 mM EDTA] in the presence of β-mercaptoethanol (10 mM) and protease inhibitors [PMSF (1 mM), leupeptin (1 μg/mL), and pepstatin A (1 μg/mL)]. The protein concentration of each extract was measured using the Bradford assay (*30*), and 100−200 μg of protein from each sample was boiled in SDS buffer [62 mM Tris-HCl (pH 6.8), 10% glycerol, 5 mM β-mercaptoethanol, and 3% SDS] and then run on a 10% polyacrylamide gel for 2.5−3 h. After semidry transfer of the protein to a nitrocellulose filter (MSI), the filter was blocked with TBST buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20] containing 5% nonfat dry milk for 2 h. The filter was then probed with a rabbit polyclonal Swi6 antibody (1:700) and a goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:4000). The protein bands were detected using the ECL Western blotting detection system from Amersham.

Band Shifts. The plasmid pBD972 was used for in vitro translation of Swi4. It contains a 700 bp CITE (*31*, *32*) and myc tag sequence insertion 5′ of the *SWI4* ATG codon and was constructed by inserting the *SWI4* gene into pBD939, which is a pBSKS+ vector with the CITE and myc tag sequence insertions between the *Apa*I and *Eco*RI restriction sites. To generate the wild-type Swi6 in vitro translation construct, pBD972 was used as a template for primers BL139 (5′CTCGAGTTCCATGGTTGTGGCC3′) and the M13 reverse primer to generate a 700 bp fragment containing the CITE sequence. After the PCR product was cloned into the pCRII vector (Invitrogen) (pBD2158), the CITE fragment was released by *Xho*I digestion and substituted for the 300 bp *Xho*I fragment of pBD1378, generating the wild-type *SWI6* CITE construct, pBD2090. Mutant allele *swi6−405* (pBD2091) and *swi6−406* (pBD2094) CITE constructs were



showing positions of residue substitutions. (A) Eleven PCR-generated temperature-sensitive mutants were isolated from the
). (B) The second group of mutants (screen 2) was isolated from both the 0.25 and the 0.1 mM $MnCl_2$ libraries. Only one
, was isolated from this screen. It was isolated four times as a single mutation and had been previously identified as a temp
rom a parent plasmid that had been previously identified in either screen 1 or 2. The residues in parentheses indicate isolat
n the additional mutations originally identified in the parent plasmid (described in the text). (C) Mutants *swi6−420*, *−421*,
e 0.5 mM $MnCl_2$ library for less severe temperature-sensitive mutants with the expectation that these would contain fewe
R344G substitution was isolated from this screen. The arrows at the top of the figure indicate single-residue substitution
bers at the bottom indicate the position of these mutations within the Swi6 protein sequence.

0.5 mM $MnCl_2$ library after
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422, and −423 and R344G
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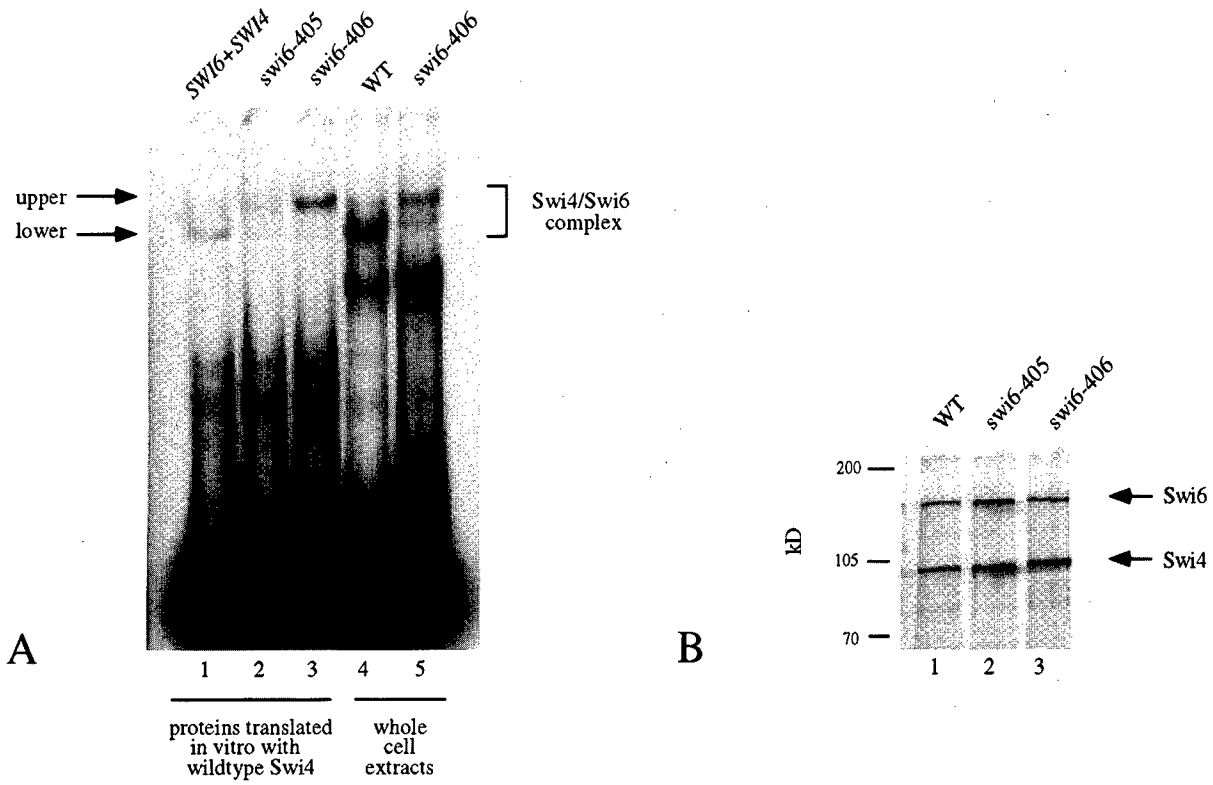

FIGURE 3: Band-shift comparison of *SWI6+* and mutant alleles using both in vitro translated proteins and whole cell extracts. (A) This band-shift gel shows comparable band-shift patterns with regard to upper and lower complex formation between a whole cell extract derived from a wild-type strain (lane 4) and in vitro translated Swi4 and Swi6 protein products (lane 1). A 130 bp fragment of the *HO* promoter, −503 to −374, was used as the DNA probe for the band-shift experiment which was performed at 25 °C. Lanes 1−3 contain proteins that were translated in vitro. Lanes 4 and 5 contain proteins expressed in whole cell extracts. Lane 1: Swi4 (pBD972) and Swi6 (pBD2090). Lane 2: Swi4 and *swi6−405* (pBD2091) (mutant protein showing both the upper and lower complex at 25 °C). Lane 3: Swi4 and *swi6−406* (pBD2094) (mutant protein showing a predominantly upper complex). Lane 4: wild type [BY600 transformed with *SWI6+* (pBD1378)]. Lane 5: *swi6−406* (pBD2046) expressed on a low-copy plasmid in the *swi6*Δ strain, BY600. (B) Both Swi4 and Swi6 are produced at comparable levels after in vitro translation (described in Experimental Procedures) at 30 °C. Lane 1: *SWI6+* (pBD2090). Lane 2: *swi6−405* (pBD2091). Lane 3: *swi6−406* (pBD2094). All other Swi6 mutants show similar expression levels in this reaction.

parallel with those produced from whole cell extracts. The resulting band-shift patterns are qualitatively similar [compare lanes 1 and 3 of Figure 3A (in vitro translation products) to lanes 4 and 5 (whole cell extracts)]. Since the in vitro translated Swi4 and Swi6 were the only yeast proteins added to the reaction mixtures in lanes 1−3, it is unlikely that the tendency of the *swi6−406* ankyrin repeat mutant to form upper complexes is due to binding of additional proteins to the DNA−protein complex. It is also unlikely that differences in protein modification are responsible, since the proteins are translated in a rabbit reticulocyte lysate system. The possibility that proteins in this lysate could bind or modify the yeast protein−DNA complex cannot be excluded, but to produce these results, the protein has to be specific for the swi6 mutant complexes. The protein would also need to be present in both rabbit and yeast cells, because the band shift is identical whether the mutant protein is translated in vitro or harvested from yeast.

To observe the extent of variation in DNA binding activity, the collection of temperature-sensitive ankyrin repeat mutants of Swi6 were translated in vitro and surveyed for DNA binding activity at two temperatures. Most of the Swi6 mutants retained the ability to complex with Swi4 and bind SCB elements at the nonpermissive temperature. Frequently, however, the mobility of the ankyrin mutant complex was noticeably altered (Figure 4) and migrated as an upper complex that could not be distinguished from that of *swi6−406*. The wild-type (*SWI6+*) band-shift pattern shows the predominant lower band at 25 °C and a more dispersed pattern, including both upper and lower complexes, at 37 °C. From the band-shift assay, the *swi6* mutants can be grouped into four categories. The first group of mutants [*swi6−406*, *−407* (G347D), and *−401*] all form the upper complex predominantly at both the permissive (25 °C) and nonpermissive (37 °C) temperatures. The second group of mutants [*swi6−405* and *swi6−410* (H323R), *−422*, *−421*, *−424* (R344G), *−402*, and *−417* (A477T)] form the upper complex at the nonpermissive temperature and both the upper and lower complex at the permissive temperature. The third phenotype is overall reduced binding, even at the permissive temperature, seen with mutants *swi6−401*, *−417*, and *−422*. The fourth group of mutants [*swi6−409* (N500T), *−415*, *−420*, *−423*, and *−418* (N500Y)] show no binding defect at either temperature. Most of the mutants show altered binding characteristics with a propensity to form the upper complex, which is exacerbated by elevated temperatures. A minority have reduced overall DNA binding. Formation of the upper complex therefore correlates with the loss of transcriptional activation, which is also enhanced at high temperatures. Despite the considerable variability in the DNA binding complexes formed, the upper complex that most of the mutants display migrates at a very similar position in the band-shift gel. Since this is not likely to be due to changes in protein composition or modification states,

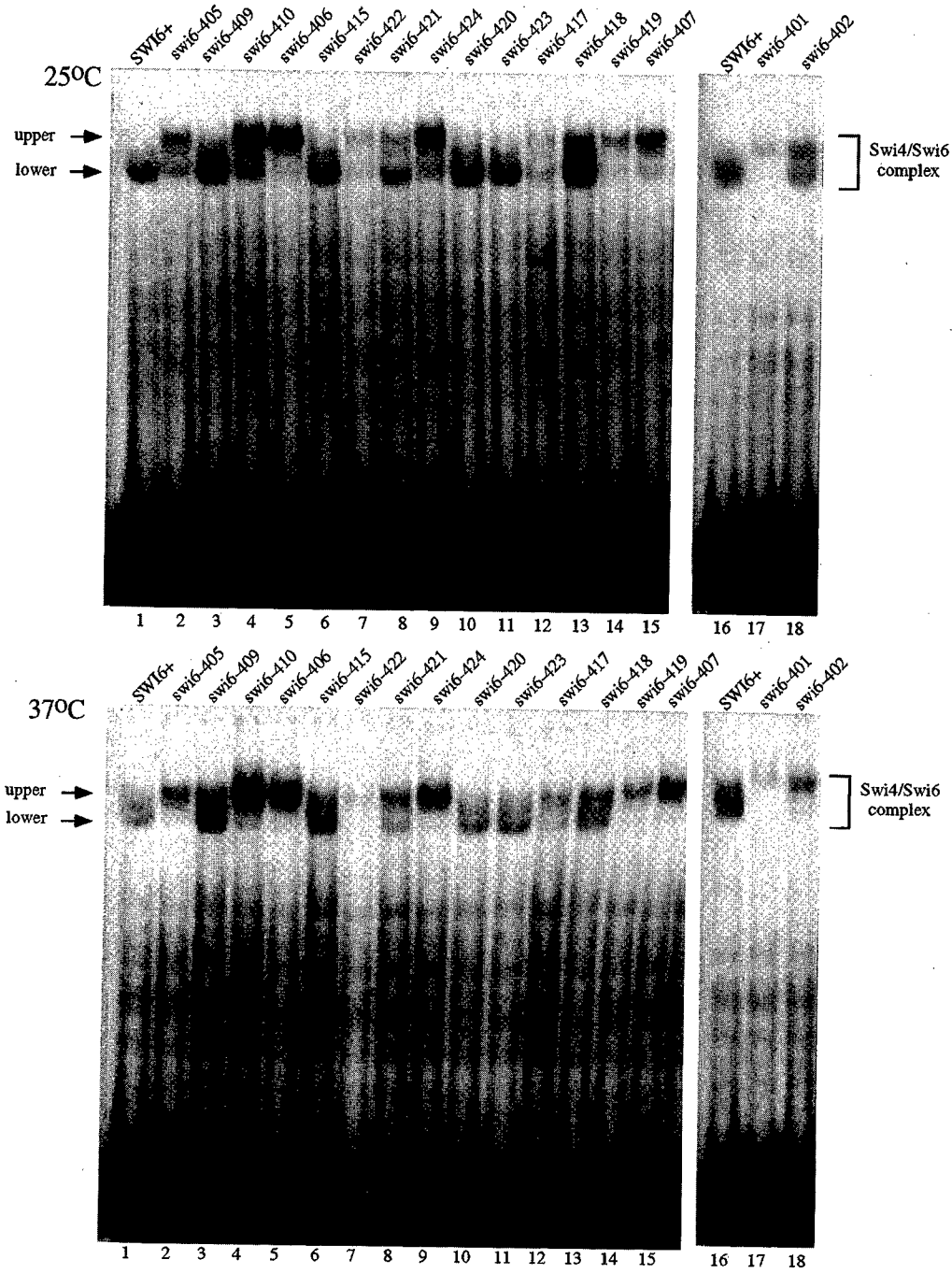

FIGURE 4: Band-shift patterns of in vitro translated wild-type and temperature-sensitive mutant *swi6* alleles. *SWI4* (pBD972) and the Swi6 plasmid indicated were translated in vitro and then used in band-shift reactions that were performed at either 25 °C (upper panel) or 37 °C (lower panel). The DNA target was a 130-base pair fragment of ^{32}P-labeled *HO* promoter containing three SCB elements. Five microliters of in vitro translation reaction product was included in each binding reaction mixture. Lanes 1 and 16: *SWI6*+ (pBD2090). Lane 2: *swi6−405* (pBD2091). Lane 3: *swi6−409* (pBD2092). Lane 4: *swi6−410* (pBD2093). Lane 5: *swi6−406* (pBD2094). Lane 6: *swi6−415* (pBD2095). Lane 7: *swi6−422* (pBD2096). Lane 8: *swi6−421* (pBD2097). Lane 9: *swi6−424* (pBD2098). Lane 10: *swi6−420* (pBD2099). Lane 11: *swi6−423* (pBD2100). Lane 12: *swi6−417* (pBD2101). Lane 13: *swi6−418* (pBD2102). Lane 14: *swi6−419* (pBD2103). Lane 15: *swi6−407* (pBD2104). Lane 17: *swi6−401* (pBD2105). Lane 18: *swi6−402* (pBD2106).

we speculate that it may be the result of a global change in the conformational state of the Swi4−Swi6 complex which these mutations induce to varying degrees.

Effects of Alanine Substitutions in Core Residues of the Ankyrin Repeats of Swi6. To assess the importance and function of the individual ankyrin repeats, and because the significance of the middle repeats was still a question, we created mutations at equivalent sites in each of the four repeats. These mutations, substitution of alanines for each of the core residues (G-T-L in repeats 1 and 4, G-S-L in repeat 2, and N-T-L in repeat 3), eliminated the three most conserved residues in the repeats. In so doing, we hoped to completely disrupt the function of the conserved residues in each repeat in a similar manner. In addition, we created various combinations of the individual core region substitutions to determine whether any of these combinations would have additive effects. Immunoblots were performed on these strains as described earlier. Each of these site-directed mutants produces a detectable Swi6 protein product at 37 °C, although, like those of the PCR-generated mutants, the

Table 2: β-Galactosidase Activities for Swi6 Mutant Yeast Strains[a]

(strain) *SWI6* allele	repeats with core residues substituted	units of *ho:lacZ* activiity as a percentage of wild-type activity	
		25 °C	37 °C
(BY1379) *SWI6+*	–	34.0 (100)	23.0 (100)
(BY2220) *swi6−1000*	1	34.0 (100)	4.2 (18)
(BY2221) *swi6−200*	2	5.0 (15)	<0.15 (0)
(BY2222) *swi6−30*	3	1.5 (5)	<0.15 (0)
(BY2223) *swi6−4*	4	<0.15 (0)	<0.15 (0)
(BY2214) *swi6−1234*	1, 2, 3, 4	23.0 (70)	4.9 (21)
(BY2215) *swi6−123*	1, 2, 3	4.5 (14)	<0.15 (0)
(BY2216) *swi6−124*	1, 2, 4	4.6 (14)	<0.15 (0)
(BY2217) *swi6−134*	1, 3, 4	2.0 (6)	<0.15 (0)
(BY2218) *swi6−234*	2, 3, 4	4.2 (13)	<0.15 (0)
(BY2219) *swi6−23*	2, 3	5.8 (18)	1.6 (7)
(BY600) *swi6*Δ	–	<0.15 (0)	<0.15(0)

[a] β-Galactosidase levels for the site-directed mutants were determined using the *ho:lacZ* reporter construct. Each mutant was analyzed at least twice in duplicate, and results were calculated as a percentage of wild-type activity.

protein levels tend to be lower than that of the wild-type control. One surprising observation is that the mutants containing alanine substitutions within multiple cores attain a higher level of Swi6 protein than those mutants in which a single core region is alanine substituted (data not shown).

Using an integrated *ho:lacZ* reporter construct, we performed β-galactosidase assays to determine the transcriptional activity of these site-directed mutants. All of the core mutants displayed a temperature-sensitive phenotype in our most sensitive X-gal filter assay (*21*) of *ho:lacZ* expression (data not shown), and these results were confirmed by quantitative assays (Table 2). The four mutants that contain only one alanine-substituted core were all defective in *ho:lacZ* expression at the nonpermissive temperature. In the context of the native *HO* promoter, the fourth repeat appears to be the most critical for *HO*, or SCB-driven, transcription, showing severely defective transcriptional activity at both permissive and nonpermissive temperatures. There is no suggestion that any one of these repeats performs a function that is redundant. In addition, the fact that all of these mutants are temperature-sensitive suggests that the conserved residues within each repeat play critical roles in stabilizing the native structure of the Swi6 protein.

Another surprising property, revealed by combining these core substitution mutants, is that the *swi6−1234* allele, which contains mutations within the core residues of all four ankyrin repeats and was expected to be the most defective, has the highest level of β-galactosidase activity when compared with the other site-directed mutants (70% at 25 °C and 21% at 37 °C) using the *ho:lacZ* reporter construct. In contrast, substitutions within only the fourth core produce a highly defective protein, with no discernible β-galactosidase activity at the same temperatures. Apparently, substitutions within the three additional core regions produce a stabilizing or compensatory effect. The addition of substitutions within the first, second, and third core regions in varying combinations with the fourth also appears to increase activity compared to that seen with the single fourth core substitution at the permissive temperature, but not to the same extent as *swi6−1234*. One plausible explanation of this high level of activity could be that the ankyrin repeats are targets of negative regulation. In that case, loss of all four repeats would eliminate all targets of this repressive activity and would result in increased *ho:lacZ* expression. We cannot exclude this possibility, but we have monitored *ho:lacZ* transcription through the cell cycle in the *swi6−1234* strain and have found it to be cell cycle-regulated (data not shown). This indicates that negative regulation during the cell cycle still persists.

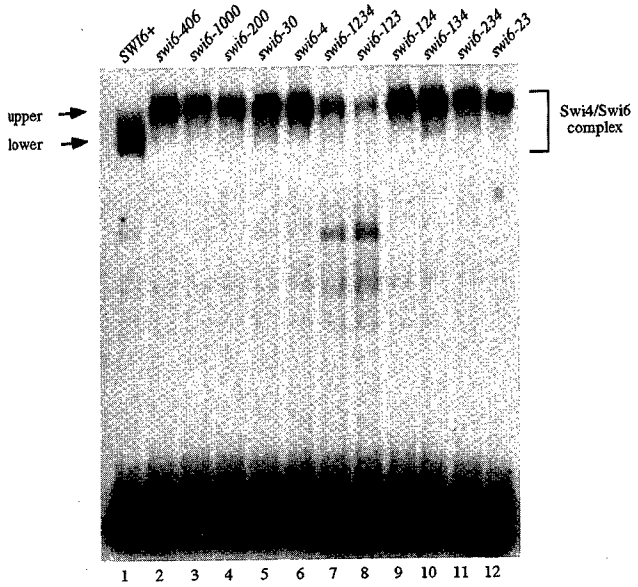

FIGURE 5: All Swi6 site-directed mutants bind SCB DNA elements in complex with Swi4 and form an exclusively upper band-shift complex. Band-shift experiments with in vitro translated Swi4 and Swi6 reveal the typical wild-type (WT) complex (lane 1), which shows the predominant lower complexes at 25 °C as indicated by the bracketed region. PCR-generated mutant *swi6−406* (lane 2) confers only the upper complex, as do all of the Swi6 site-directed mutants. The band-shift results shown are from a 25 °C binding experiment carried out as described in Figure 4. While the wild-type complex reveals a more distinct lower band at 25 °C, there was no difference in the Swi6 mutant band-shift results between the permissive and nonpermissive temperatures. Lane 1: *SWI6+* (pBD2090). Lane 2: *swi6−406* (pBD2094). Lane 3: *swi6−1000* (pBD2159). Lane 4: *swi6−200* (pBD2160). Lane 5: *swi6−30* (pBD2161). Lane 6: *swi6−4* (pBD2162). Lane 7: *swi6−1234* (pBD2163). Lane 8: *swi6−123* (pBD2164). Lane 9: *swi6−124* (pBD2165). Lane 10: *swi6−134* (pBD2166). Lane 11: *swi6−234* (pBD2167). Lane 12: *swi6−23* (pBD2168).

To measure the DNA binding defect associated with the core substitution mutants, we employed in vitro translation and band-shift assays as before, incubating the reaction mixtures at both 25 and 37 °C. As stated above, the wild-type Swi4−Swi6 complex exists as a combination of upper and lower complexes on SCB DNA, with a predominant lower complex at 25 °C. The core mutants all form the upper complex exclusively at both temperatures (Figure 5, 25 °C experiment shown). The ability to form the upper band-shift complex did not localize to mutations within a particular core region. Thus, even more so than with the PCR-generated repeat mutants, we see a dramatic but uniform shift in mobility of the DNA binding complex in the core mutants of Swi6. This, and the temperature sensitivity of all these mutants, suggests that the conserved core residues of all four repeats are important for maintaining the native structure of Swi6 and/or the Swi4−Swi6 complex.

Differential Effects upon SCB- versus MCB-Directed Transcription. We originally isolated the PCR-generated mutants because they were defective in transcription from the predominantly SCB-driven *ho:lacZ* promoter, so it was

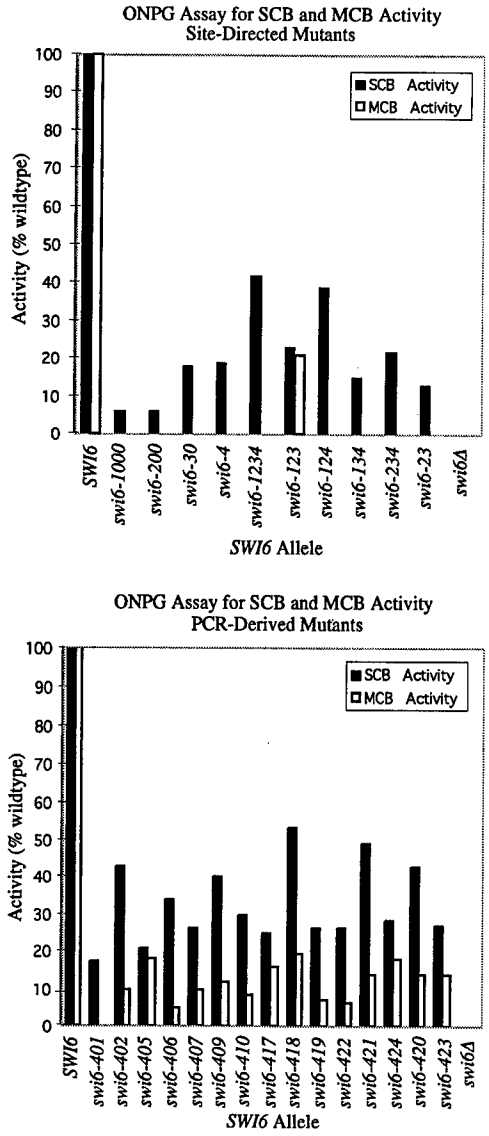

FIGURE 6: ONPG assay results for SCB and MCB reporter constructs. Using three tandem SCB (CACGAAAA) or MCB (ACGCGT) DNA binding elements driving the *LacZ* reporter, we measured the transcriptional activity (details in Experimental Procedures) of selected temperature-sensitive mutants. The activity is reported as a percent of the wild-type activity. The wild-type *SWI6* gene and each of the mutant alleles were on a low-copy plasmid in the presence of the reporter construct, which was expressed from a 2 μm plasmid.

of interest to measure the activities of these mutant proteins in MCB-driven transcription, which is activated by Swi6 bound to another partner, Mbp1 (*5*, *6*, *10*). To allow a direct comparison between SCB and MCB activation, we introduced a high-copy reporter construct carrying three tandem repeats of either MCB or SCB elements into the BY660 (*ho, swi6::TRP1−197*) strain carrying the mutant *swi6* allele on a low-copy plasmid (Figure 6). This enabled us to compare SCB and MCB activation within an equivalent context. Figure 6 shows that most of the PCR-derived and site-directed mutants cause a more severe MCB transcriptional defect when compared with SCB transcriptional activity. The most notable differences are seen with *swi6−1234*, which has 44% activity on SCBs and 0% on MCBs, *swi4−124*, which has 40% SCB activity and 0% MCB activity, and *swi6−421*, which has 48% of the wild-type SCB activity compared with 14% of the wild-type MCB activity. In addition, there are a number of mutants (*swi6−1000*, *−200*, *−123*, *−405*, *−417*, and *−424*) that show comparably decreased levels of both SCB and MCB activity when measured from these analogous promoter constructs. The fact that the vast majority of ankyrin repeat mutants are not equivalently defective with respect to MCB and SCB activation indicates there are differential effects upon the Swi4−Swi6 and Mbp1−Swi6 transcription complexes. This is somewhat surprising since the C termini, and not the ankyrin repeats of these proteins, are sufficient for the interaction between Swi6 and Swi4 (*3*, *4*) and between Swi6 and Mbp1 (*9*). Interestingly, all of the mutants are more defective in MCB activation than in SCB activation, despite the fact that they were selected for their inability to act at the *HO* promoter, which is activated by SCB elements. This suggests that the Swi6−Mbp1 interaction or activity has a stronger dependence either upon the ankyrin domain itself or upon other residues that are brought into proper position by the ankyrin domain structure.

Modeling Studies of the Ankyrin Repeat Mutations. The recent publication of the crystal structure of a p53 binding protein, 53BP2, which contains four ankyrin repeats (*17*), has enabled us to estimate the positions of the ankyrin repeat mutations of Swi6 that we have generated. First, the four ankyrin repeats of Swi6 and 53BP2 were aligned with 24 other ankyrin repeat domains using CLUSTAL-W (*36*). This multiple alignment provided a reliable means of aligning the two proteins' ankyrin repeats despite their low sequence identity (21%). Then, the coordinates for the 53BP2 structure, generously provided by N. P. Pavletich, were used in conjunction with the MODELLER program (*37*) to generate and refine a model of the Swi6 ankyrin domain. This model was strikingly similar to the 53BP2 structure. It was validated by three independent methods [PROCHECK, VERIFY-3D, and ERRAT (see the Figure 7 legend)], and all three indicated a high degree of reliability. The structure that 53BP2 adopts, and the one that the modeled Swi6 conforms to, resembles an "L" in profile, with continuous, tightly packed α-helices perpendicular to and connected by short β-hairpins. A ribbon diagram of the Swi6 ankyrin model structure is provided in Figure 7. The locations of the core residues and five single-point mutants are labeled. The regularity of this repeat structure is apparent in this view, as is the conserved positioning of the core residues in each repeat.

Alanine substitutions were made in the three most conserved core residues of the Swi6 ankyrin repeats in an effort to completely eliminate whatever function this repeating and conserved structure might have. These substitutions clearly disrupt Swi6 function, but because there are three changes in the structure in each repeat, it is not possible to be certain which of these substitutions is the most disruptive. However, sequence comparison with other ankyrin repeat-containing proteins, as well as inspection of the model of the Swi6 structure, suggests that the third substitution within the cores, which substitutes alanine for leucine at positions L322, L355, L392, and L474, is the most disruptive. First, while the glycine and threonine are conserved within the Swi6 family, these specific residues are not conserved in the ankyrin repeat family as a whole. In contrast, the leucine is highly conserved among the hundreds of ankyrin repeats that have been identified (*15*). In addition, inspection of the

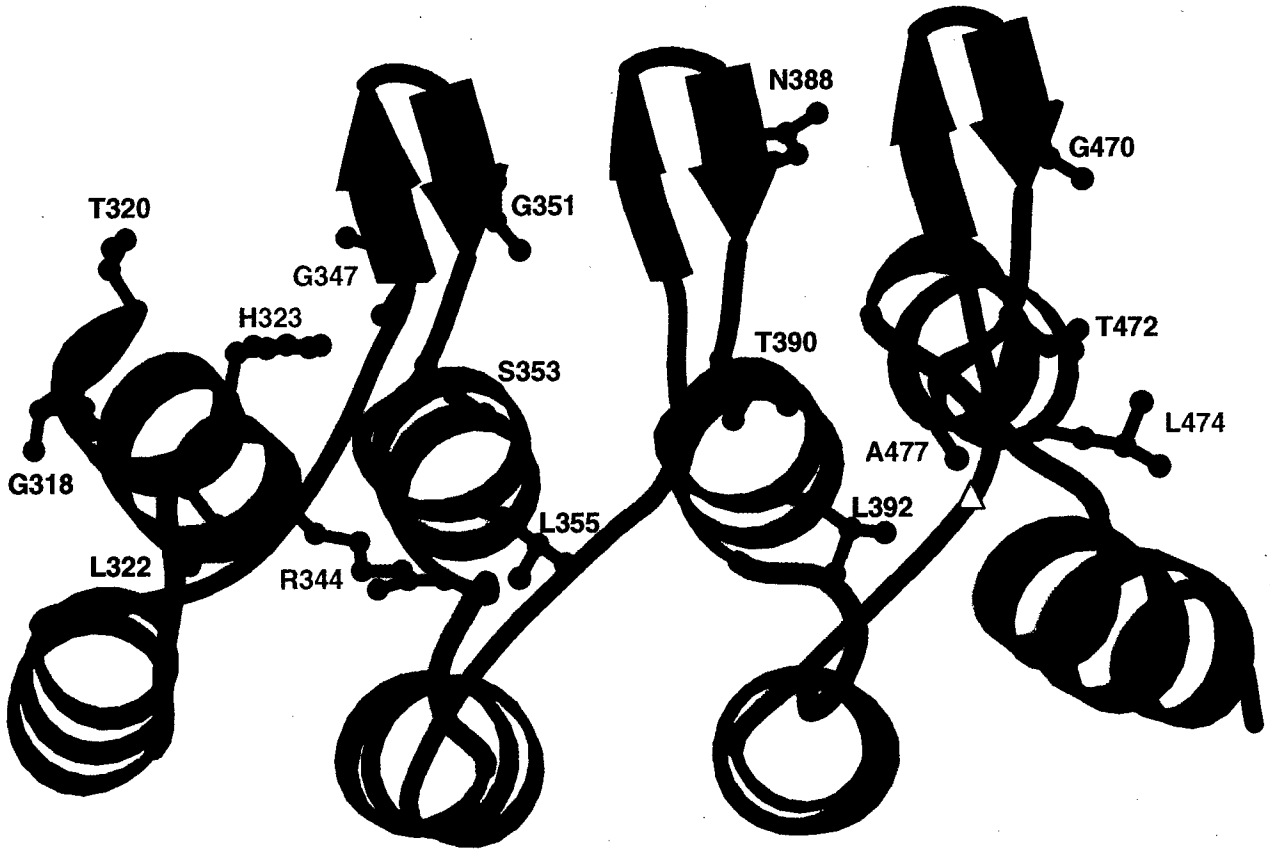

FIGURE 7: Ribbon diagram of the Swi6 ankyrin domain model. The model consists of three and one-half ankyrin repeats with the β-L-β-L-α-L-α motif. Here α-helix, β-strand, and loop (L) are color coded with blue, red, and green, respectively. The highly conserved core residues in the ankyrin repeats, G318, T320, L322, G351, S353, L355, N388, T390, L392, G470, and T472, and the single-site mutants generated in this study, H323, R344, G347, L474, and A477, are highlighted with ball-and-stick models. The single-site mutants are labeled in cyan, and the core residues are labeled in black, except for L474, which is a core residue that was also identified as a single mutant in our temperature-sensitivity screen. The color coding of the atoms is as follows: carbon, black; nitrogen, blue; and oxygen, red. Only the side chain atoms for each residue are shown in the ball-and-stick model except glycine where the main chain atoms are shown for easy identification. The model of the Swi6 ankyrin domain was derived from the crystal structure of a homologous ankyrin domain in the p53-binding protein (*17*) by the homology modeling method (*37*). Twenty-four sequences of ankyrin repeat domains were aligned by CLUSTAL-W (*36*) to achieve a reliable alignment of the Swi6 ankyrin domain with that of the p53-binding protein. The multiple alignment was required due to the low sequence homology between the ankyrin domains in Swi6 and the p53-binding protein. The initial model was generated and subsequently refined by MODELLER (*37*). The final model presented here was validated by PROCHECK (*45*), VERIFY_3D (*46*), and ERRAT (*47*). The result of PROCHECK showed that 99.1% of the main chain dihedral angles are within allowed regions. The rmsd bond length and angle are 0.02 Å and 2.47°, respectively. The average 3D_1D profile score for each residue is 0.34, with the lowest score of 0.24 for the N- and C-terminal residues as calculated from VERIFY_3D. ERRAT showed that 96.0% of the residues are within the 95% confidence limit. There are no residues above the 99% confidence limit level. These three independent validation methods suggest that the model is reliable. The long insertion between repeats 3 and 4 (residues 418−462) was excluded in the model, and a yellow triangle denotes the position of that undefined stretch in the structure.

Swi6 model shows that these leucines are part of a hydrophobic core which is likely to play a critical role in stabilizing the four α-helix bundles that are the predominant feature of the Swi6 ankyrin domain. The first of these three core leucines, L322, lies at the interface of four α-helices and is spaced appropriately to form tight hydrophobic interactions with three other hydrophobic residues in the adjacent helices (V334, V338, and L373). Leucine is often found to be a preferred residue in leucine zippers, coiled coils, and other structures in which α-helices are tightly packed (*38*), and in ankyrin repeats, they may also play a critical role in stabilizing the helix bundles.

Four ankyrin repeats occur in tandem within the yeast transcription factor family, and the four full repeats have been modeled on the basis of the structure of another protein which also contains four repeats. N500, one of the residues shown to be critical for Swi6 function in this analysis, is adjacent and C-terminal to the four-repeat structure. This asparagine, and the glycine that follows three residues downstream, are the residues that would be expected to begin a fifth repeat. However, the residues that follow do not conform to the consensus sequence. The significance of this partial fifth repeat is unclear, but the fact that it maintains appropriate spacing and sequence conservation within this family suggests that it may be important in the overall domain structure. This is corroborated by the fact that point mutations in N500 have been found which disrupt Swi6 function. In addition, there is another ankyrin repeat containing protein in yeast, Yar1, which also ends its ankyrin repeat sequence in the same manner (*39*).

Among the PCR-generated ankyrin repeat mutants, the most commonly identified mutation, isolated 10 times from three independent screens, was at the glycine G347 position and all were substitutions of glycine for aspartate (D). G347, as pictured in Figure 7, lies on the back surface of the modeled structure, in the β-hairpin connecting ankyrin

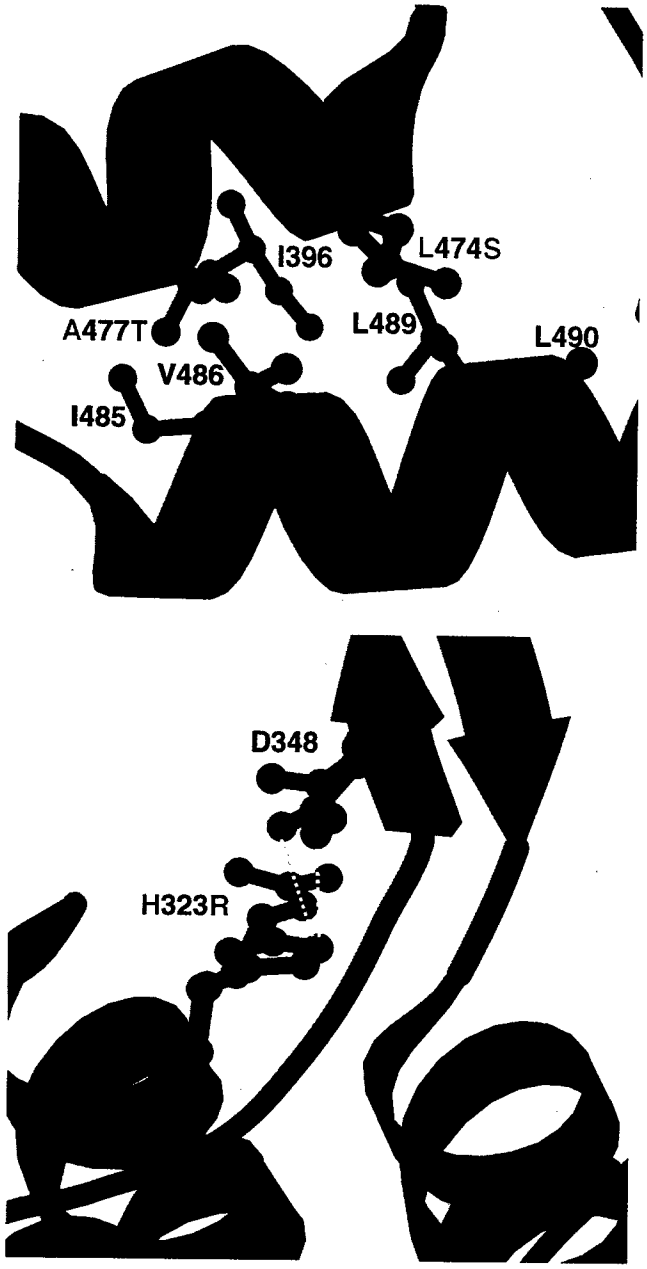

FIGURE 8: (A, top) Superposition of the two models of the L474S and A477T mutants where L474 and A477 in the wild type are substituted by Ser and Thr, respectively. The positions of mutant residues S474 and T477 were generated by MODELLER and subsequently refined. L474 and A477 and their neighboring residues, I396, I485, V486, L489, and L490 in the wild type as well as S474 and T477 in the mutant, are represented with ball-and-stick models. L474 and A477 in the wild type are indicated with a cyan bond color. Their neighboring residues in the wild type are indicated with a green bond color. L474 forms tight hydrophobic packing with the side chains of V486, L489, and L490, whereas A477 forms tight hydrophobic packing with I396, I485, and V486. In both mutants, the substitution of L474 by S474 and of A477 by T477 inserts a hydrophilic residue in the hydrophobic pocket, which will destabilize the four-α-helix bundle. (B, bottom) Model of the H323R mutant where H323 is substituted by Arg. The position of mutant residue R323 was generated by MODELLER and subsequently refined. Residue 323 and the neighboring D348 in the wild type and the mutant Swi6 are represented with ball-and-stick models and coded by their bond color of cyan and purple, respectively. The $N_{\epsilon 2}$ of H323 forms two hydrogen bonds indicated by yellow dashed lines with $O_{\delta 1}$ and $O_{\delta 2}$ of D348 in the wild type, whereas the guanidinium group of R323 in the mutant clashes with D348, although the side chain of D348 was pushed away from its wild-type position. This steric hindrance may lead to thermal instability of the mutant.

repeats 1 and 2. As such, changes at that position are unlikely to destabilize the structure itself. Rather, the loss of function that this mutation causes suggests that this residue may be part of a surface on which there is a critical interaction with another protein, or with another domain of the Swi6 protein. This is also likely to be the case for the gain of function mutation, N330K, which does not confer a conformational change to the modeled Swi6 ankyrin repeat.

The four other single mutations generated in this study that resulted in temperature-sensitive Swi6 proteins are all predicted to result in unfavorable interactions within the ankyrin repeat itself when modeled onto the structure. These findings help to validate the model and give us new insight into the key intramolecular interactions that occur in this structure. The core leucine (L) at position 474 changed to serine (S), and alanine (A) 477 changed to threonine (T) (Figure 8A); both disrupt the tight hydrophobic packing that is predicted to occur within neighboring residues. In the case of histidine (H) 323 (Figure 8B), which is replaced with arginine (R), the hydrogen bonds predicted to be formed by the histidine with aspartate (D) 348 are disrupted and the larger arginine side chain would be expected to perturb the local structure due to steric hindrance. Finally, the space filling models depicted in panels A and B of Figure 9 clearly show the effect predicted by substitution of R344 for glycine (G). In the model, the loss of the bulky side chain of the arginine creates a cavity on the surface of the structure which exposes the hydrophobic side chains of L322 and L373. This cavity is large enough to make this region accessible to water and would be expected to destabilize the structure.

DISCUSSION

Within the four ankyrin repeats in this family of transcription factors, three levels of conservation are observed. There are "core" residues, which are identical, as well as residues with chemical similarities that are conserved throughout all four of the repeats. Second, there are specific residues which are shared by repeats 1 and 4, making them much more similar to each other than the other repeats in these or any other set of ankyrin repeat proteins. Third, there are residues which are conserved within the individual repeats of all of the family members, but which differ widely between repeats. This study provides evidence that the core residues of all four of the repeats are important, but whether this is due to a structural requirement for a four-repeat domain or whether these repeats actually have different roles is unclear. The fact that the mutations in the core residues all result in temperature sensitivity and all cause what appears to be a dramatic conformational change in the Swi4−Swi6 DNA binding complex is evidence that the role of these residues may be structural.

Modeling studies of our single-point mutants also show that all but one of these mutations are likely to disrupt the structure of the ankyrin repeat domain. The one exception is G347D, which may define a surface on which there is a critical interaction. This interaction is most likely to be between Swi6 and Swi4, or with another part of Swi6, as this mutant also causes a dramatic shift in the ternary complex between Swi4, Swi6, and DNA on band-shift gels. Our transcript measurements show that all of the ankyrin repeat mutants, which were initially selected for defects in

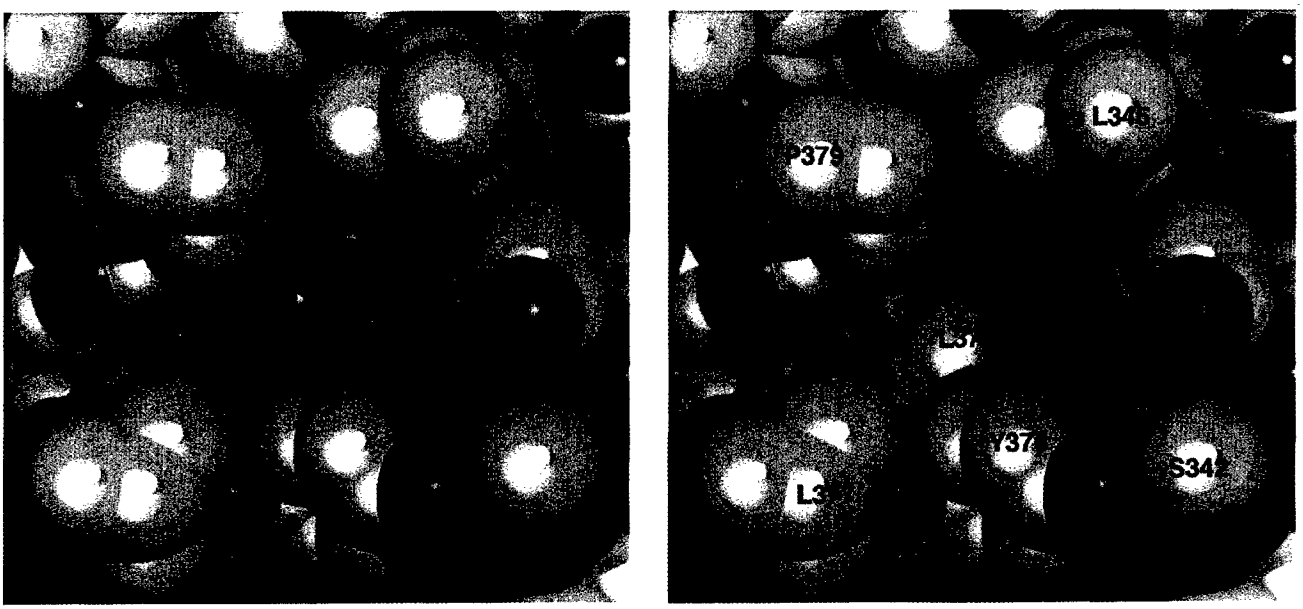

FIGURE 9: Space filling models of the wild-type Swi6 ankyrin domain in the region of R344 (A, left) and the mutant in the same region where R344 was replaced by G344 (B, right). The position of mutant residue G344 was generated by MODELLER and subsequently refined. The standard van der Waals radii were used to generate the spheres that represent all the atoms. The color coding is as follows: carbon, light green; nitrogen, blue; and oxygen, red. Panels A and B are viewed from the same orientation. Residue R344 in the wild-type Swi6 is labeled in panel A. Residue G344 in the Swi6 mutant and the neighboring residues are labeled in panel B. The substitution of R344 by G344 created a cavity in the mutant Swi6 and exposed the hydrophobic side chains L322 and L373. This R344G mutation will destabilize the Swi6 ankyrin domain due to the exposure of hydrophobic residues.

SCB-driven transcription in the context of the native *HO* promoter, have an even more severe defect in MCB-driven transcription. This greater dependence upon the ankyrin repeat domain for Mbp1−Swi6- than that for Swi4−Swi6-dependent transcription indicates differences in the contacts within the two complexes. These residues may be identifiable with more exhaustive genetic screens.

Ankyrin repeats were initially identified as statistically significant homologies between the repeats of Swi6 and its nearest *S. pombe* relative, Cdc10. Ankyrin repeats were also found in the Notch protein of *Drosophila* and the *Caenorhabditis elegans* lin-12 protein, both of which are also highly related (*1*). Since then, ankyrin repeats have been identified in more than 100 proteins with highly diverse functions (*15*). The sequence consensus has been relaxed considerably over that time, and the discovery of new members of the family is aided as much by the fact that they are nearly always present as tandem repeats as by their sequence similarities.

The function of the ankyrin repeat has been investigated in many different systems, and several key protein−protein interactions have been shown to depend on them (*40*−*44*). However, the repeats do not appear to interact with each other, nor has any other "signature" sequence been identified that is diagnostic of an ankyrin repeat-interacting protein. This, coupled with the fact that the proteins in which these ankyrin repeats are found have highly diverse functions and are located in a myriad of different cellular compartments (spider venoms, membrane transport proteins, and transcription factors), suggests that these repeats do not have a common function or binding partner. Rather, they should be viewed as structural units, which confer a particular type of protein fold. Gorina and Pavletich (*17*) have noted that ankyrin repeats form a novel L-shaped structure. Our study provides evidence that the conserved residues within each repeat are required to produce this structure, and as such, they provide a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues, which are unique to the different classes of ankyrin repeat-containing proteins, are responsible for specific interactions with other proteins and for providing the biological specificity and function to the ankyrin repeat proteins. The conserved residues, which are the defining feature of an ankyrin repeat, may play a strictly conformational function. Thus, the ankyrin repeat may be more appropriately viewed as a novel type of protein fold which provides a stable structure with surfaces that can be tailored for many different macromolecular interactions. Our data indicate that the ankyrin repeats of Swi6 are critical for the thermostability of Swi6 and for maintaining the proper conformation of the ternary complex between Swi4, Swi6, and DNA. It is still possible that the unique faces of the Swi6 ankyrin repeat domain form a binding site for another protein that has not yet been identified. However, it is more likely that these repeats provide a rigid structure that holds the Swi4−Swi6 complex in a precise and functional spatial arrangement with respect to the DNA.

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Appendix 2
DAMD17-94-J-4122

The *MSN1* and *NHP6A* Genes Suppress *SWI6* Defects in *Saccharomyces cerevisiae*

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ABSTRACT

Ankyrin (ANK) repeats were first found in the Swi6 transcription factor of *Saccharomyces cerevisiae* and since then were identified in many proteins of eukaryotes and prokaryotes. These repeats are thought to serve as protein association domains. In Swi6, ANK repeats affect DNA binding of both the Swi4/Swi6 and Mbp1/Swi6 complexes. We have previously described generation of random mutations within the ANK repeats of Swi6 that render the protein temperature sensitive in its ability to activate *HO* transcription. Two of these *SWI6* mutants were used in a screen for high copy suppressors of this phenotype. We found that *MSN1*, which encodes a transcriptional activator, and *NHP6A*, which encodes an HMG-like protein, are able to suppress defective Swi6 function. Both of these gene products are involved in *HO* transcription, and Nhp6A may also be involved in *CLN1* transcription. Moreover, because overexpression of *NHP6A* can suppress caffeine sensitivity of one of the *SWI6* ANK mutants, *swi6-405*, other *SWI6*-dependent genes may also be affected by Nhp6A. We hypothesize that Nhp6A and Msn1 modulate Swi6-dependent gene transcription indirectly, through effects on chromatin structure or other transcription factors, because we have not been able to demonstrate that either Msn1 or Nhp6A interact with the Swi4/Swi6 complex.

THE Swi6 protein of *Saccharomyces cerevisiae* is involved in the regulation of dozens of genes that are transcribed at the G_1/S transition of the cell cycle. These include the genes encoding the *HO* endonuclease, G1 cyclins (NASMYTH and DIRICK 1991; OGAS *et al.* 1991; DIRICK *et al.* 1992; MEASDAY *et al.* 1994), and many of the genes involved in DNA replication (MCINTOSH *et al.* 1988; LOWNDES *et al.* 1991, 1992; DIRICK *et al.* 1992). Swi6 associates with at least two DNA-binding factors, Swi4 and Mbp1. These associations are mediated by the C-terminal domains of the proteins (ANDREWS and MOORE 1992b; PRIMIG *et al.* 1992; KOCH *et al.* 1993; SIDOROVA and BREEDEN 1993), and the N termini of Swi4 and Mbp1 confer the DNA-binding specificity to these complexes. The Swi4/Swi6 complex binds to SCB (CACGAAA) elements in *CLN2, HO,* and *PCL1* (*PHO85* CycLin) and MCB-like elements of *CLN1* (ANDREWS and HERSKOWITZ 1989; ANDREWS and MOORE 1992a; PRIMIG *et al.* 1992a; PARTRIDGE *et al.* 1997). The Mbp1/Swi6 complex binds to MCB (ACGCGTnA) elements (MCINTOSH *et al.* 1991; LOWNDES *et al.* 1992; KOCH *et al.* 1993).

Swi4, Mbp1, Swi6, and the *S. pombe* homologues Res1, Res2, and Cdc10 contain four ankyrin (ANK) repeats. These repeats are degenerate 33 amino acid motifs that are found in tandem in many different proteins in both eukaryotes and prokaryotes (BORK 1993). In several cases ANK repeats have been shown to be involved in protein-protein association (THOMPSON *et al.* 1991; HENKEL *et al.* 1992; LAMBERT and BENNETT 1993; SCHNEIDER *et al.* 1994). The Swi6 ANK repeats are critical for its function, but their role is unclear. Our modeling analysis of Swi6 ANK repeats (EWASKOW *et al.* 1998) suggests that they may represent a new protein fold that supports active conformation of Swi6 complexes and/or properly displays protein association interfaces.

We have generated numerous ANK repeat mutations in Swi6 that render a transcriptionally inactive protein (SIDOROVA and BREEDEN 1993; EWASKOW *et al.* 1998). At the biochemical level, these mutations can lead to a reduced DNA binding, but most also cause a significant shift in the mobility of Swi4/Swi6 complexes in band shift gels (EWASKOW *et al.* 1998). The latter suggests the possibility that the DNA-bound Swi4/Swi6 complex can undergo a significant conformational change. Furthermore, it is possible that there are accessory proteins that may modulate this change to affect transcriptional activity of the Swi4/Swi6 complex.

In this work we used two of the temperature-sensitive ANK alleles of *SWI6* in a high copy suppressor screen to find proteins needed for the full activity of the Swi4/Swi6 complex. We report the results of this screen and the further analysis of two of these high copy suppressors, *NHP6A* and *MSN1*.

MATERIALS AND METHODS

Strains and plasmids: The BY600 *MAT***a** *swi6::TRP1 ade ho::lacZ ura3 his3 leu2-3,112 trp1-1 can1-100 met2* and BY606 *MAT***a** *swi4::LEU2 ade ho::lacZ ura3 his3 leu2-3,112 trp1-1 can1-100 met2* strains are described in SIDOROVA and BREEDEN (1993). The BY1998 strain *MAT***a** *his3Δ200 ura3-52 lys2-801*

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ade2-101 msn1Δ1::TRP1 trp1 was kindly provided by M. Carlson (ESTRUCH and CARLSON 1990). The BY2036 strain *MATα ura3-52 trp1-289 his3Δ1 leu2-3 gal2 gal10 nhp6a-Δ3::URA3 nhp6b-Δ3::HIS3* was kindly provided by M. Snyder (COSTIGAN *et al.* 1994). BY28 *MAT***a** *ade2 his3 leu2-3,112 trp1-1 ura3* is W303-1a. The plasmid pSWI6 (pBD1378) was described previously (SIDOROVA *et al.* 1995). pSWI6-406 (pBD2046) and pSWI6-405 (pBD2031) are analogous to pBD1378, but contain mutated alleles of *SWI6*. Positions of these and other mutations in the *SWI6* alleles used in this study are listed in Table 2. Strains BY1954 *swi6Δ LEU2::swi6-405*, BY1956 *swi6Δ LEU2::swi6-406*, and analogous strains bearing other ANK mutations were constructed by integrative transformation of BY600 strain (SIDOROVA and BREEDEN 1993) with linearized pRS305 plasmids carrying *SWI6* DNA (*Hin*dIII to *Sma*I fragments). High copy suppressor subclones were generated in pRS426 or pZUC12 2μ vector backgrounds. Subcloned suppressors were sequenced with M13 universal or reverse primers and library clones borne on YEp24 vector were sequenced with primer BL146 5′ACTACGCGATCATGG3′. The plasmid pBD2068 was a gift from M. Snyder and contains an HA-tagged *NHP6A* on the YEp352 vector (COSTIGAN *et al.* 1994). The plasmids pDK267 and pDK268 were provided by D. Kolodrubetz. pDK267 contains *NHP6B* sequence flanked by ~700 bp of genomic sequence on either side, cloned into *Eco*RI-*Hin*dIII-digested YEp352. pDK268 carries a 1.6-kb *Eco*RI-*Pst*I fragment containing the *NHP6A* gene and flanking sequences cloned into YEp352.

Growth conditions: All rich (YEPD) and minimal (YC) media and growth conditions were as described previously (BREEDEN and MIKESELL 1991). Temperature-sensitive ANK mutant strains were cultivated at 30° and shifted to 37° for 8–12 hr when grown in liquid media. When grown on plates, they were incubated at 37° for the whole period of growth.

DNA, RNA, and protein analysis: FACS analysis of yeast cells was done as described in (HEICHMAN and ROBERTS 1996) and data were analyzed using CellQuest software. Procedures for RNA isolation and S1 protection were performed as described previously (BREEDEN and MIKESELL 1991). Protein extract preparation, immunoprecipitation, and Western blotting were done as described before (SIDOROVA and BREEDEN 1993; SIDOROVA *et al.* 1995).

***In vitro* transcription and translation:** The plasmid pBD972 was used for *in vitro* translation of Swi4 (EWASKOW *et al.* 1998). pBD972 was added to a TNT rabbit reticulocyte lysate coupled transcription translation system (Promega, Madison, WI) along with 20–50 ng of the recombinant Swi6 purified from *Escherichia coli* (SIDOROVA and BREEDEN 1993). Reactions were carried out according to manufacturer's recommendations with cold amino acids. Reaction products were added directly to *HO* promoter DNA-binding reactions or loaded onto SDS PAGE.

Gst fusion and purification from yeast or bacterial cells: To construct *GST* fusions, *MSN1* and *NHP6A* were generated by polymerase chain reaction (PCR) from pM-4 (pBD2050) and pN-5 (pBD2055), respectively, using M13 reverse primer and BL138 5′GGATCCATGGTCACCCCAAGAG3′ primer for *NHP6A*, and M13 reverse and BL137 5′CCGGATCCATGG CAAGTAACC3′ primers for *MSN1*. PCR fragments were cloned into pCRII (Invitrogen, Carlsbad, CA) generating pBD2056 and pBD2059, respectively. For construction of the *GST-NHP6A* fusion, the 2.3-kb *Bam*HI fragment with the *NHP6A* open reading frame out of pBD2056 was cloned into *Bam*HI-digested pBD1905, which bears *GST* under the control of *GAL* promoter in pRS316 vector, to give rise to pBD2057, or into *Bam*HI-cut pGex2T (Pharmacia, Piscataway, NJ) to make pBD2064. The *GAL-GST-NHP6A* cassette was also recloned into a 2μ vector pBD2055 by insertion of the 2.9-kb *Eco*RV fragment of pBD2057, containing a portion of the *URA3* gene and *GAL-GST-NHP6A*, into *Eco*RV-cut pBD2055; the resulting construct is called pBD2063.

For the *GST-MSN1* fusions (pBD2061 and 2062), first the 1.5-kb *Sac*I fragment from pBD2049 was substituted for the *Sac*I fragment in pBD2058. Then the 1.4-kb *Bam*HI fragment of the resulting pBD2060 containing *MSN1* was cloned into *Bam*HI-cut pBD1905, to give rise to pBD2061, or into *Bam*HI-cut pGex2T (Pharmacia), to give rise to pBD2062.

To purify Gst fusions from *E. coli*, pBD2064 and pBD2062 were transformed into DH5α cells and the resulting strains were treated according to Pharmacia Biotech Gene Fusion System protocols. Bacterial cultures were grown to OD 0.6, and fusion protein expression was induced by 0.1 mM isopropyl thiogalactoside for 2 hr. Cells were then harvested, sonicated, centrifuged, and extracts were incubated with glutathione Sepharose 4B beads (Sigma, St. Louis) for 30 min at 4°. To determine if the Gst fusions were capable of interacting with Swi6, these glutathione beads with fusion proteins immobilized on them were incubated with recombinant Swi6 or *in vitro*-translated Swi4/Swi6 complex, washed, boiled, and loaded onto SDS PAGE.

To obtain Gst fusions from yeast, pBD2057, pBD2063, and pBD2061 were transformed into W303-1a strain. The resulting strains were grown in selective media with raffinose overnight and then expression of the fusions was induced by galactose for 3–4 hr. Cells were harvested, and protein extracts were prepared as described before (SIDOROVA *et al.* 1995) and incubated for 1 hr with glutathione beads in GST buffer containing protease inhibitors (100 mM Tris HCl pH 8.0, 100 mM NaCl, 0.2% NP40 with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Beads were then washed in three or four changes of GST buffer. To elute fusion proteins from the beads, the beads were resuspended in 50 ml of glutathione buffer, prepared according to the Pharmacia Biotech protocol. Fusions were eluted for 15 min at room temperature. To determine if Swi6 copurified with any of the Gst fusions from yeast, glutathione eluates were loaded onto SDS PAGE, and Western blots were performed with Swi6 antibodies. Alternatively, extracts of yeast cells expressing fusion proteins were subjected to immunoprecipitation with Swi6 or Swi4 antibodies. Immunoprecipitates were loaded onto SDS PAGE, Western blotted, and probed with Gst antibodies (Santa Cruz).

Thrombin cleavage of the Nhp6A from the Gst-Nhp6A fusion, bound to glutathione beads, was performed according to Pharmacia Biotech protocols. Glutathione beads were mixed with 38 μl of PBS and 2 μl of thrombin solution (1 unit/μl thrombin in PBS), incubated overnight at room temperature, and centrifuged. Supernatants were used directly for DNA-binding reactions.

Gel retardation: Gel retardation analysis was performed exactly as described (SIDOROVA and BREEDEN 1993; EWASKOW *et al.* 1998). When the *in vitro*-translated Swi4/Swi6 complex was bound to DNA, little (0.2–0.5 μg) or no nonspecific competitor dI-dC was added. The binding pattern was the same, regardless of whether dI-dC was present in the reaction or not. Thrombin or glutathione eluates of the Gst-Nhp6A fusion were directly added to DNA-binding reactions with *HO* promoter fragment. No dI-dC competitor was used in these reactions since Nhp6A is a nonspecific DNA binder (PAULL and JOHNSON 1995) and can be competed from *HO* DNA by dI-dC (J. SIDOROVA, unpublished results).

RESULTS

Screen for high copy suppressors of temperature-sensitive *ho::lacZ* expression phenotype of *swi6-405* and

TABLE 1

High copy suppressors of *SW16* ankyrin repeat mutants

Plasmid	Suppressor	Suppression of *ho::lacZ* expression in strains with the following mutations		
		swi6-406	*swi6Δ*	*swi4Δ*
	A. Suppressors isolated from *swi6-406* strain			
c1 (5)	*SW16*	+	+	NA[a]
c2a (1)	ND[b]	+	+	NA
c4 (1)	*MSN1*	+	±	−
c6 (1)	ND	+	+	NA
	B. Suppressors isolated from *swi6-405* strain			
c1 (1), c14 (2)	*SW16*	+	+	NA
c2 (1)	ND	+	±	−
c4 (8), c5 (1), c9 (1), c12 (1)	*MSN1*	+	±	−
c15 (1)	*NHP6A*	+	−	−
c19 (1)	ND	+	±	±
c23 (1)	ND	+	±	−

The strains indicated above were transformed with vectors or with vectors bearing suppressing genes, grown on plates at 37°, and screened for β-galactosidase activity with X-gal assays. Numbers in parentheses by the clone names indicate the number of times this clone was isolated.

[a] Not applicable.

[b] Not determined.

***swi6-406* mutants:** We have previously carried out random mutagenesis of the ANK repeat-encoding region of the *SWI6* gene (EWASKOW *et al.* 1998). Two of these mutants, *swi6-405* (N330T, N500Y) and *swi6-406* (T326I, T402S), were used in this study. Both mutants express Swi6 protein at the nonpermissive temperature; however, the level of Swi6 is reduced as compared to the wild-type protein level (Figure 2A). There is no *ho::lacZ* activity detected in *swi6Δ* strains expressing Swi6-405 or Swi6-406 from the CEN plasmid pRS316 at 37°, but at 30 and 25°, they confer partial activity as judged by cell morphology and *ho::lacZ* transcript levels (data not shown and see below). In band shift assays, Swi4/Swi6-405 complex is less active in binding to SCB elements than the wild-type complex, and Swi4/Swi6-406 complex has an altered mobility (EWASKOW *et al.* 1998).

The mutated *SWI6* genes were integrated at the *LEU2* locus of the *swi6Δ ho::lacZ* strain, giving rise to strains BY1954 (*swi6-405*) and BY1956 (*swi6-406*). These strains were transformed with a 2μ-based yeast genomic library (CARLSON and BOTSTEIN 1982) and about 60,000 transformants were obtained for each. Colonies were grown at 30° for the first 2 days upon transformation and then incubated at 37° overnight. Colonies were transferred to nitrocellulose filters and assayed for β-galactosidase activity using the X-gal filter assay (BREEDEN and NASMYTH 1985). Transformants that developed blue color above the background were selected. Library plasmids were isolated out of these cells, retransformed into BY1954 or BY1956 strains, and reassayed to confirm suppression. They were also transformed into BY600 *swi6Δ ho:lacZ* strain to determine if candidate suppressors were able to bypass the Swi6 function.

We recovered a total of 8 suppressor plasmids from *swi6-406* and 19 from *swi6-405* transformants. Using restriction digestion and PCR, we identified four different suppressors for *swi6-406* and six for *swi6-405*. These are listed in Table 1. The *SWI6* gene was isolated five times in the screen with *swi6-406* and three times with *swi6-405*. Two suppressors (c2a and c6) were not pursued further because they activated *ho::lacZ* expression equally strongly in *swi6-405* and *swi6Δ* cells and thus were completely independent of Swi6. Most of the suppressors (c2, c4, c5, c9, c12, c19, and c23) suppressed the *ho::lacZ* expression defect to some extent in the absence of Swi6, but only one (c19) could suppress in the absence of Swi4. This requirement for Swi4 suggests that the majority of suppressors enhance Swi4-mediated activation, rather than cause a general derepression of transcription. c15 showed no suppression of the *ho::lacZ* transcription defect in *swi4* or *swi6* deletion strains and thus was the best candidate for an allele-specific suppressor.

Sequence information was obtained for all the Swi4- and Swi6-dependent suppressors. c4, c5, c9, and c12 carry overlapping fragments from chromosome *XV*, and c23 has a nonoverlapping fragment from the same chromosome. c15 carries a fragment from chromosome *XVI*. Finally, c2 has a fragment from chromosome *XIII*. In each case more than one open reading frame was present on the insert. In this article we describe identification and further analysis of the genes responsible for suppression by c15 and c4.

We have subcloned fragments of c4 and c15 into pRS426 and transformed the resulting constructs into *swi6-406* or *swi6-405* to determine which of the open

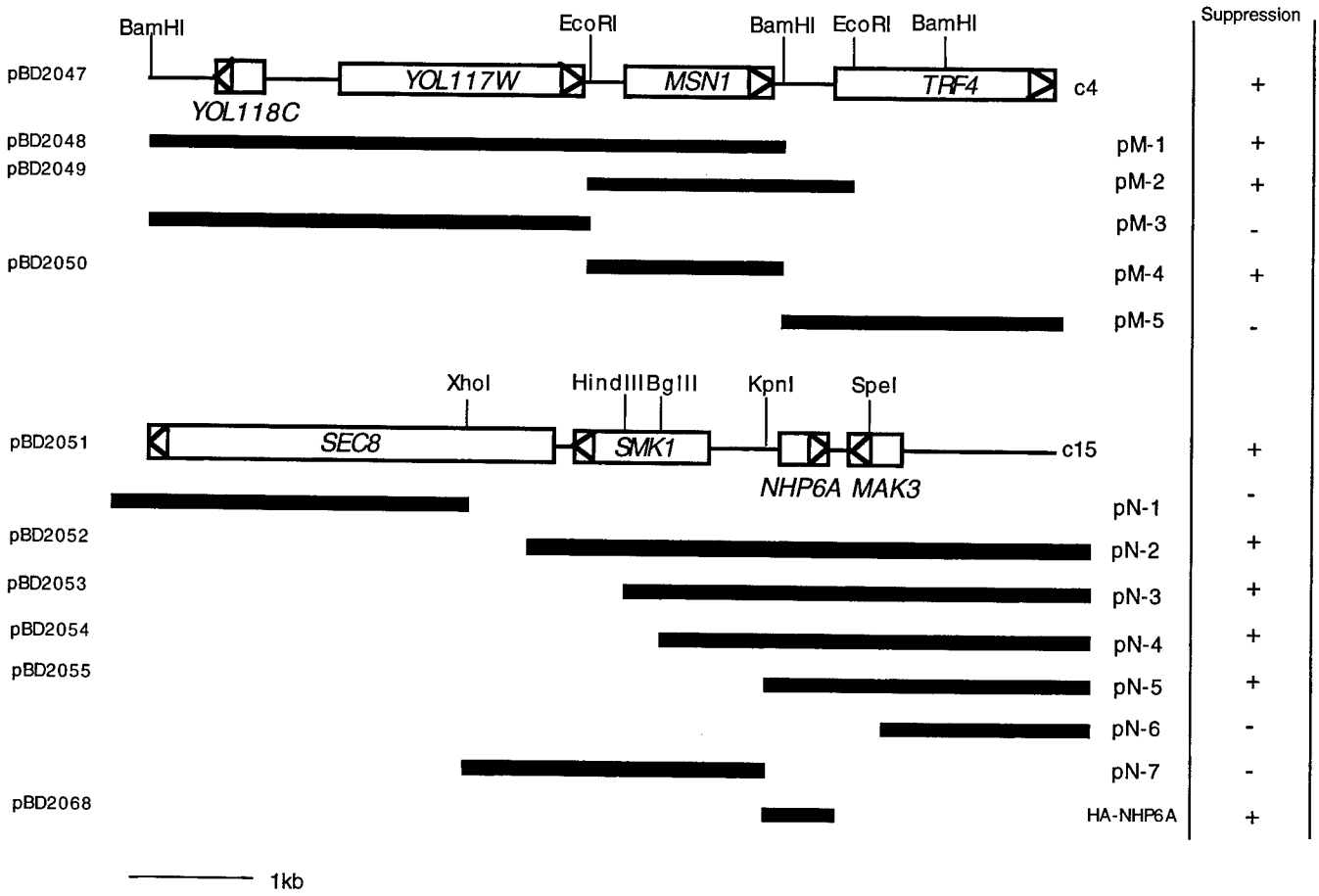

FIGURE 1.—*MSN1* and *NHP6A* suppress *SWI6* mutants. Shown are the maps of suppressors c4 and c15 and the subclones generated to identify the open reading frames responsible for suppression. Triangles within boxes show direction of transcription of the genes. The names of the subclones generated in this study are listed on the right; M stands for *MSN1* and N for *NHP6A*. The 1-kb segment below designates the scale. The subclones of c4 and c15 were transformed into BY1956 *swi6-406* and BY1954 *swi6-405*, respectively, streaked onto selective media plates, and grown at 37°. Then X-gal filter assays were performed. BY1954 and BY1956 transformed with pRS426 or pZUC12 served as negative controls for these assays. The development of blue color above the negative control level was scored as suppression.

reading frames encode suppressors. The results of mapping and subcloning are summarized in Figure 1. Previously identified genes, *MSN1* and *NHP6A*, were responsible for the suppression phenotypes of c4 and c15, respectively. *MSN1* was originally cloned as a high copy suppressor of a temperature-sensitive *SNF1* kinase mutant for its ability to restore *SUC2* expression (ESTRUCH and CARLSON 1990). It acts as a transcriptional activator when fused to LexA and does not have any specific DNA-binding activity (ESTRUCH and CARLSON 1990). *NHP6A* has also been identified previously (KOLODRUBETZ and BURGUM 1990) and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of bending DNA (PAULL and JOHNSON 1995).

***MSN1* and *NHP6A* suppress the *HO* transcription defect of *SWI6* ANK mutants:** Since *NHP6A* (c15) was incapable of bypassing Swi6 function, we sought to determine if *NHP6A* suppression was specific to the *swi6-405* allele of *SWI6*. The *NHP6A* gene on pRS426 was transformed into strains with different mutant alleles of *SWI6*, and X-gal filter assays were performed. All these mutants express Swi6 at nonpermissive temperature (37°; EWASKOW *et al.* 1998; and data not shown). *swi6-Δ21* encodes a nonconditional and highly defective Swi6 protein (SIDOROVA and BREEDEN 1993). As seen in Table 2, the temperature-sensitive *ho::lacZ* expression phenotype of many of the ANK mutants, including *swi6-406*, could be suppressed by elevated levels of *NHP6A*. The one exception was *swi6-401*, which is the most defective mutant of the set tested. In addition, *NHP6A* could not suppress the *ho::lacZ* expression defect of *swi6-Δ21*, which carries a deletion of the putative leucine zipper in *SWI6*. Thus, *NHP6A* displays allele-specific suppression. It enhances transcription by some temperature-labile Swi6 proteins and has no detectable suppressing activity with others. This could indicate a direct interaction between Nhp6A and Swi6, which is disrupted by only a subset of the Swi6 mutants. However, because Nhp6A suppresses all but the most defective alleles of *SWI6*, this could also be explained if there is a threshold

TABLE 2

NHP6A* is an allele-specific suppressor of *SWI6

Strain number	Allele	Mutation	ANK repeat affected	Suppression by *NHP6A*
BY1954	*swi6-405*	N330T	1	+
		N500Y	4	
BY1956	*swi6-406*	T326I	1	+
		T402S	3	
BY1957	*swi6-402*	A329T, K339R	1	±
		I395T	3	
BY1958	*swi6-401*	K357E	2	−
		N469I, A494G	4	
BY1959	*swi6-A1*	T326I, I328L	1	+
		L453S	Spacer	
		K501R	4	
BY1960	*swi6-407*	G347D	2	+
BY1693	*swi6-Δ21*	D(E585-L606)	None	−
BY2223	*swi6-4*	G470A, T472A, L474A	4	+

The original residue, its position, and the substituting residue describe mutations (for example N330T is glutamine at position 330 in the Swi6 protein changed to threonine). The subdivision of the ANK region into four full copies of the repeats is as in BREEDEN (1996). The spacer region is a stretch of amino acids between the third and fourth repeats of Swi6 that is not conserved among the members of the Swi4/Swi6 family (BREEDEN 1996). Plasmid pN-5 bearing *NHP6A* gene was transformed into the strains carrying indicated mutant *SW16* alleles. These transformants were then grown at 37° (or at 30°, in the case of *swi6Δ-21*) on plates and subjected to X-gal assays. Strains transformed by pRS426 vector served as controls for these assays.

for detection of suppression and some mutants fall below this threshold.

β-Galactosidase assays show that suppression by the 2μ plasmid-borne *NHP6A* or *MSN1* of the *ho::lacZ* expression defect in *swi6-405* or *swi6-406* cells at 37° is low but well above background. For example, β-galactosidase activities for *swi6-406* transformed with *MSN1* (pM-2) or *NHP6A* (pN-5) are 22 and 15 units, respectively, compared to 5 units for the vector-transformed control. To see if this increased expression occurred at the transcription level, we analyzed levels of *ho::lacZ* mRNA in these strains by S1 protection. Both at 25 and 30°, the *ho::lacZ* mRNA level was noticeably higher in the *swi6-405* and *swi6-406* strains transformed with high copy *NHP6A* and *MSN1* plasmids, respectively, as compared to the same strains carrying the vector alone (Figure 2, B and C). At 37°, *ho::lacZ* mRNA level in these strains was too low to be reproducibly quantitated by S1 protection even in the presence of suppressors (data not shown). A similar result was obtained when *MSN1* plasmid was transformed into *swi6-405* (Figure 2D). These data show that both *MSN1* and *NHP6A* exert their function at the mRNA level, rather than by affecting β-galactosidase stability or activity.

Nhp6A has a close homologue, Nhp6B, which has a set of properties indistinguishable from Nhp6A. The two proteins may have overlapping functions, because only deletion of both genes has a discernible phenotype (COSTIGAN *et al.* 1994). However, *NHP6B* was not among the suppressors that we isolated. Thus, we tested *NHP6B* directly for suppression of *SWI6* ANK mutations. When expressed from a high copy vector (pDK267), *NHP6B* also suppresses *swi6-405*. It is a weaker suppressor than *NHP6A* (pDK268, Figure 2B), but this difference may be due to the lower levels of *NHP6B* expression, which could also explain why different *NHP6A*-expressing plasmids suppress the *ho::lacZ* mRNA transcription defect to slightly different degrees (Figure 2B).

Swi6-405 and Swi6-406 are maintained at lower levels than the wild-type protein, so one indirect mechanism of suppression by *NHP6A* and *NHP6B* could be that of increasing expression of *swi6-405*, *swi6-406*, or the *SWI4* gene. To test this possibility, we looked at *SWI4* transcription in the *swi6-405* strains with or without the elevated level of Nhp6A and found no difference in *SWI4* mRNA levels (data not shown). We were not able to test if the Swi4 protein levels were affected, because available antibodies do not detect endogenous levels of this protein. However, because Nhp6A and Nhp6B are generally considered to be involved in DNA metabolism rather than in protein stability, the fact that *SWI4* transcript levels are not affected by Nhp6A overproduction makes it likely that the protein levels are also unaltered. We also measured the levels of Swi6-405 protein at 37° in cells transformed with vector alone or with *NHP6A*- or *NHP6B*-expressing plasmids and found that the mutant Swi6 accumulated to the same level in all strains tested (Figure 2A). Thus, the suppression by Nhp6A or Nhp6B proteins cannot be attributed to the increase in *SWI4* or *SWI6* expression.

***MSN1* and *NHP6A* are involved in *HO* transcription:** To determine if *MSN1* and *NHP6A* are normally in-

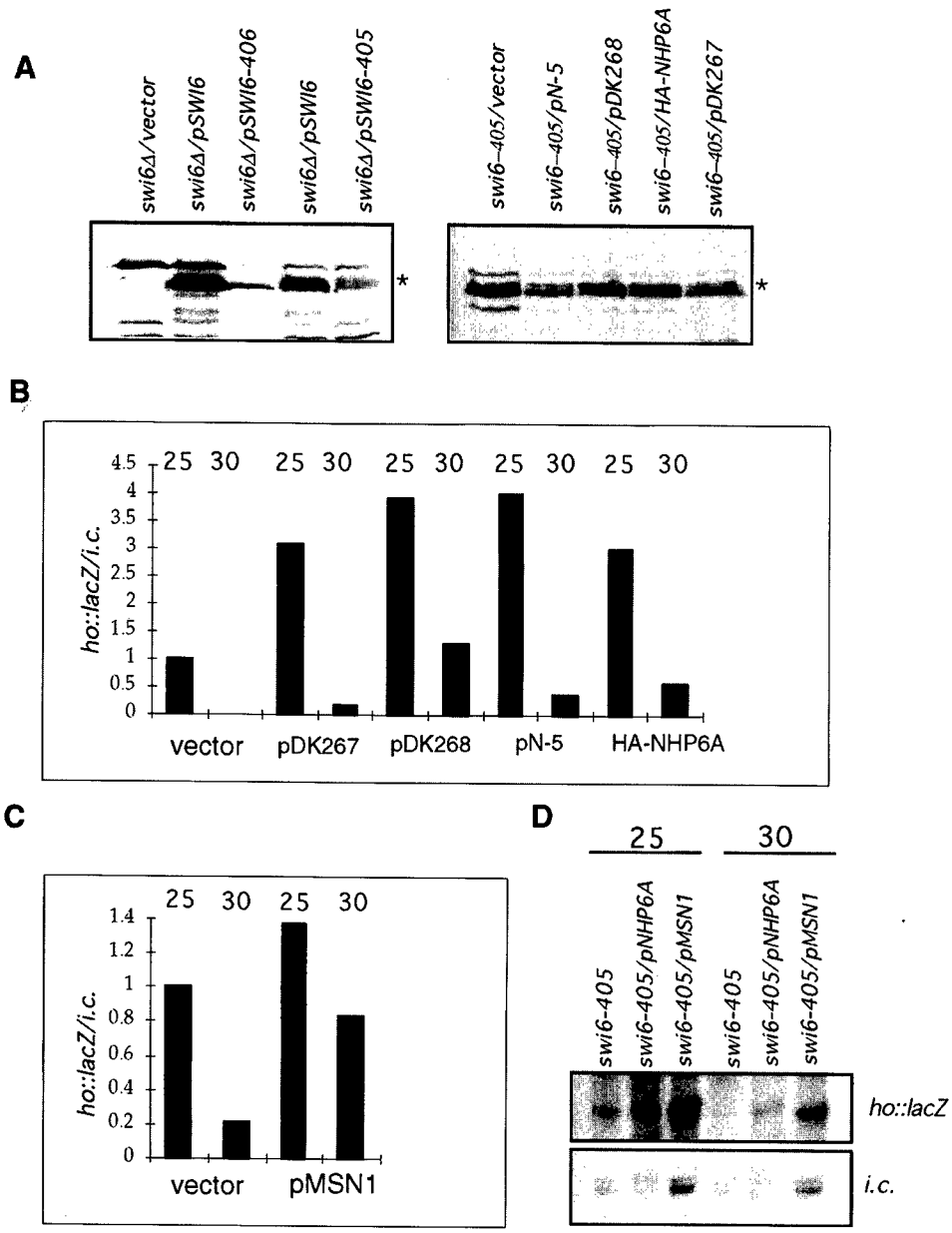

FIGURE 2.—*NHP6A* and *MSN1* suppress *swi6-405* and *swi6-406* ANK mutant *ho::lacZ* transcription defects. (A) Western blots of Swi6 expressed in the following strains at 37°. BY600 was transformed with pRS316 (vector), pSWI6 pBD1378, pSWI6-405 pBD2031, and pSWI6-406 pBD2046 and BY1954 *swi6-405 ho::lacZ* strain was transformed with the vector pRS426 or *NHP6A*-carrying plasmids pN-5, pDK268, HA-NHP6A and with *NHP6B*-carrying pDK267 (see MATERIALS AND METHODS and Figure 1). An asterisk marks the position of the Swi6 protein. (B) S1 protection was performed on RNAs isolated from the same set of BY1954 *swi6-405* strains grown at 25° or 30° for 10 hr. Levels of *ho::lacZ* mRNA obtained from two to three measurements were quantitated, normalized to the internal control levels (*SIR3* mRNA), and plotted. (C) S1 protection was performed on RNAs isolated from the BY1956 *swi6-406* strain transformed with vector pRS426 or *MSN1*-carrying pM-2 and grown for 10 hr at 25° or 30°. *ho::lacZ* levels were measured and quantitated as in B. (D) S1 protection was performed on BY1954 *swi6-405* strain transformed with vector pRS426, pN-5, or c12 (an *MSN1*-carrying clone) and grown at 25° or 30°. Positions of *ho::lacZ* and internal control (*SIR3*) transcripts are marked.

volved in the transcription of the Swi4/Swi6-regulated promoters, we isolated mRNA from exponentially growing cultures of strains with or without *MSN1* or *NHP6A* and *NHP6B* gene products and compared the levels of *HO* and *CLN1* transcripts in these strains to the wild-type strain by S1 protection (Figure 3). The *msn1Δ* strain expresses about three- to fivefold less *HO* transcript and the *nhp6abΔ* strain shows a twofold drop in *HO* transcript compared to wild type. Interestingly, there is little or no effect of *msn1Δ* on another Swi4/Swi6-regulated promoter, *CLN1* (data not shown), but the *nhp6abΔ* has a similar twofold effect on *CLN1* (Figure 3, B and C).

***NHP6A* and *NHP6B* genes can suppress the caffeine sensitivity of the *swi6-405* allele:** The *NHP6A* gene has been implicated as a downstream target of the Slt2/Mpk1 MAP kinase pathway that leads from Pkc1 and is involved in growth control and cell morphogenesis (COSTIGAN *et al.* 1994), in part because overexpression of *NHP6A* or *NHP6B* suppresses several Slt2 pathway defects, including the caffeine sensitivity of *slt2* mutants. Because *swi6Δ* mutants are also sensitive to caffeine (IGUAL *et al.* 1996), we examined whether the *SWI6* ANK mutant *swi6-405* is sensitive to caffeine, and, if so, whether this defect can be suppressed by overexpression of *NHP6A* or *NHP6B*. The *swi6-405* strain cannot grow at 37° on plates containing 4.5 mM caffeine, whereas the wild-type cells were capable of growing on these plates (data not shown). However, when *NHP6A* and *NHP6B* genes expressed from high copy plasmids were transformed into the *swi6-405* strain, they restored the ability of these cells to grow on plates containing up to 5.5 mM caffeine (Figure 4A).

Nhp6A probably affects Swi4/Swi6 DNA binding indirectly: To see whether there is a direct interaction be-

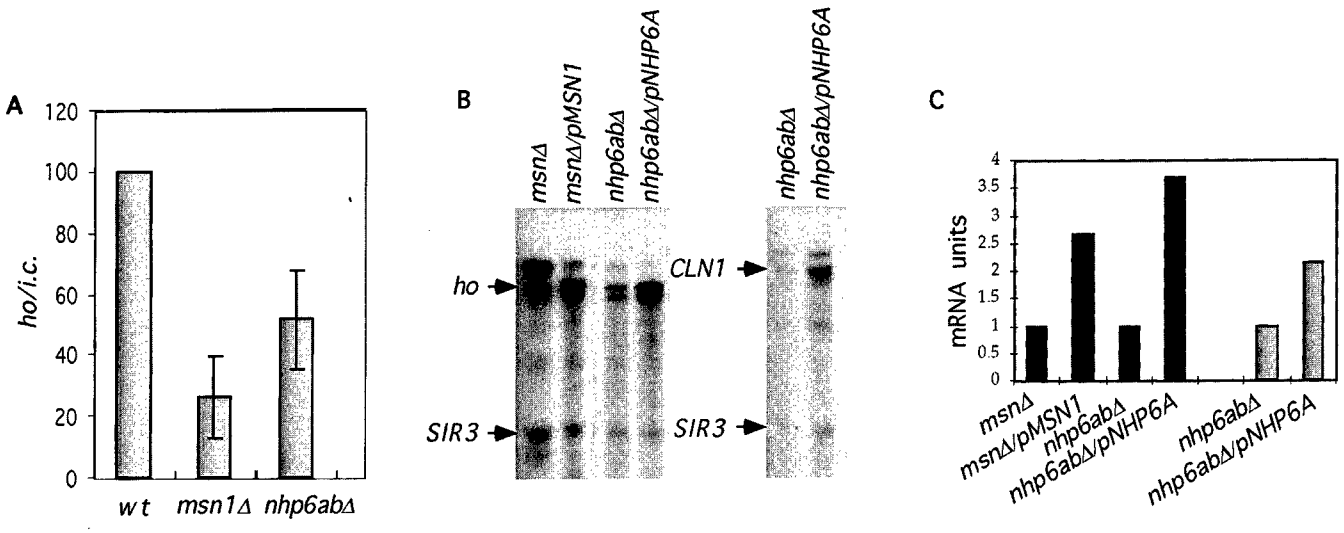

FIGURE 3.—*HO* expression is reduced in *msn1*Δ and *nhp6ab*Δ strains. (A) Five or six S1 protection measurements of the steady-state levels of *HO* mRNA were made, quantitated, and normalized to the internal control message (*SIR3* RNA). In each independent experiment, the wild-type W303-1a strain measurement was equaled to 100% and the measurements obtained for *msn1*Δ and *nhp6ab*Δ strains were calculated as the percentage of the wild type, and then averaged. The error bars show the average deviation of these measurements. (B) The *msn1*Δ strain was transformed with vector pRS426 (lane 1) or high copy *MSN1* (pM-1) (lane 2), and *nhp6ab*Δ was transformed with pBD2076 [a pSH144 Guarente vector version with a *LEU2* marker (EWASKOW *et al.* 1998; lane 3)] or high copy *NHP6A* (pN-3) (lane 4). The levels of *HO* or *CLN1* in the resulting strains were measured by S1 protection. *SIR3* mRNA serves as internal control. (C) Levels of *HO* or *CLN1* were normalized to the levels of the internal control and plotted. The normalized levels of *HO* in *msn1*Δ strains and *HO* and *CLN1* in *nhp6ab*Δ strains were set equal to 1 unit and the transcript levels found in the same strains transformed with *MSN1*- or *NHP6A*-bearing plasmids are expressed in these units. Black bars represent *HO* message levels and gray bars correspond to *CLN1* message levels.

tween Nhp6A and Swi6, we immunoprecipitated Swi4 or Swi6 proteins out of wild-type extracts carrying HA-Nhp6A and then immunoblotted with anti-HA antibodies to detect HA-Nhp6A. Despite the fact that this HA-tagged Nhp6A is functional and can suppress the *swi6-405* transcription defect (Figure 2B), there was no indication that HA-Nhp6A coprecipitates either with Swi4 or Swi6 under the same conditions that we use to detect Swi4/Swi6 association (SIDOROVA and BREEDEN 1993).

We then prepared fusions of Nhp6A and Msn1 proteins with Gst. The *GST-NHP6A* and *GST-MSN1* fusions were put under the control of *GAL1-10* promoter and expressed in a wild-type strain. When plated under conditions which select for the plasmid and cause overexpression of the fusion, neither one of these strains was able to form colonies (Figure 4B), indicating that overexpression of either Msn1 or Nhp6A as Gst fusions is lethal. Liquid cultures of the same strains were grown in raffinose and then incubated with galactose for up to 8 hr. FACS profiles of these cultures did not show accumulation of cells in any single compartment of the cell cycle; thus overproduction of Gst-Msn1 or Gst-Nhp6A did not lead to a specific cell cycle arrest (data not shown).

Because of their toxicity, we purified these Gst fusions from cells grown in raffinose and then induced by galactose addition for only 3–4 hr. Though we could purify the fusion proteins from these cells under low-stringency conditions, there was no detectable Swi6 copurifying with either of them, as we judged by probing the fusion protein isolates with Swi6 antibodies on Western blots. We also purified these Gst fusion proteins from *E. coli* on glutathione beads and then incubated the fusion-bound beads with recombinant Swi6 (SIDOROVA and BREEDEN 1993) or *in vitro*-translated Swi4/Swi6 complex (EWASKOW *et al.* 1998). Even under these conditions, we could not detect interaction between Swi4, Swi6 on one side and the fusion proteins on the other (data not shown).

Nhp6A may not directly associate with Swi6, yet it could facilitate the binding of the Swi4/Swi6 complex to SCBs by inducing a favorable bend in DNA. Thus, we tested the involvement of Nhp6A in the Swi6 complex formation on DNA. Band shift analysis was carried out with Swi4/Swi6 complexes obtained by *in vitro* translation of Swi4 in the presence of recombinant Swi6. These complexes appeared to be identical to the complex observed in whole cell extracts, in that they migrated with the same mobility, contained both Swi4 and Swi6 proteins, as judged by their supershifting by Swi4 and Swi6 antibodies, and were specific to SCB elements of the *HO* promoter fragment (Figure 5A and data not shown). The purified Gst-Nhp6A fusion was first tested in gel retardation assays with the *HO* promoter fragment. The Gst-Nhp6A fusion, but not Gst alone, was able to nonspecifically bind DNA (Figure 5B, lane 8).

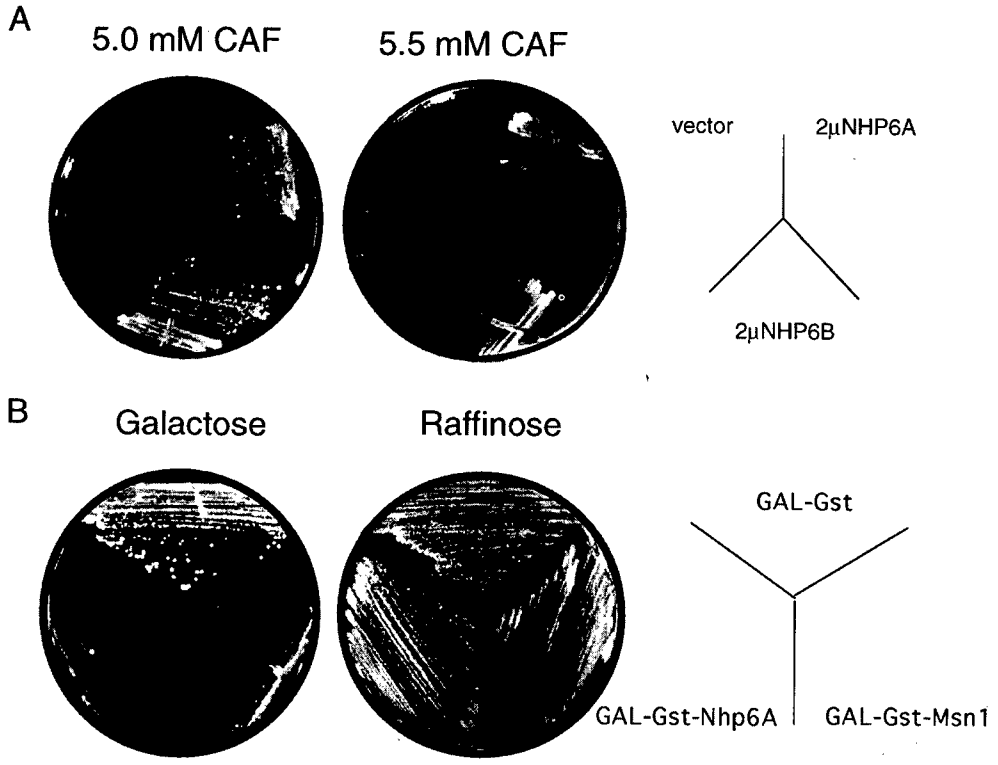

FIGURE 4.—(A) *NHP6A* and *NHP6B* suppress the caffeine sensitivity of the *swi6-405* allele. BY1954 *swi6-405* strains transformed with the pRS426 vector, pDK267 or pDK268, carrying *NHP6B* and *NHP6A*, respectively, were streaked onto YC-ura plates containing 5.0 or 5.5 mM caffeine and allowed to grow for 5–6 days at 37°. (B) Overproduction of Gst-Msn1 or Gst-Nhp6A fusions is toxic to the cells. pBD1905, pBD2061, and pBD2065, expressing Gst, Gst-Msn1, or Gst-Nhp6A from the *GAL1-10* promoter, were transformed into a W303-1a strain, grown on selective media plates with glucose and then streaked onto fresh plates with raffinose or galactose as indicated.

We also cleaved the Gst moiety off the Gst-Nhp6A fusion with thrombin and tested the released Nhp6A in gel retardation assays. As anticipated, thrombin cleavage of Gst-Nhp6A released a DNA-binding component that forms a much smaller complex on DNA (Figure 5B, lane 15). However, both Gst-Nhp6A and Nhp6A were able to bind DNA and both could form a series of band-shifts indicating that multiple Nhp6A molecules bound simultaneously to one DNA molecule. Next, the *in vitro*-translated Swi4/Swi6 complex was mixed together with varying amounts of Gst-Nhp6A or Nhp6A and was added to the *HO* promoter fragment (Figure 5B, lanes 5–7 and 12–14). These reactions were compared to the ones in which Swi4/Swi6 complex was mixed with Gst only (lanes 1–3), thrombin cleavage mixture only (lanes 9–11), and Gst-Nhp6A or Nhp6A mixed with the rabbit reticulocyte lysate (lanes 8 and 15). The amount of the DNA-bound Swi4/Swi6 complex was unaffected by the addition of Gst-Nhp6A (compare lanes 1–3 with 5–7) and slightly reduced upon the addition of Nhp6A (compare lanes 9–11 and 12–14). These results indicate that there is no cooperation between Nhp6A and Swi4/Swi6 in binding to DNA under the conditions of this assay. It is also worth noting that there appeared to be a negative effect on the amount of DNA-bound Gst-Nhp6A and Nhp6A if the Swi4/Swi6 complex was present in the reaction (compare lanes 7 to 8 and 14 to 15). Because the *HO* DNA was in excess in the reaction, and the Swi4/Swi6 complex was at subsaturating level, this cannot be attributed simply to competition for binding sites.

DISCUSSION

In this article we describe a screen for genes that are able to suppress ANK defects of Swi6 when expressed at high copy levels. Two of these genes, *MSN1* and *NHP6A*, were further characterized. Both *NHP6A* and *MSN1* are weak suppressors of *swi6* ANK mutant alleles. Both of the gene products are required for maximal expression of *HO*, as mutations in these genes lead to a two- and threefold drop in *HO* mRNA levels, respectively. However, neither protein appears to directly associate with Swi6.

MSN1 (also named *FUP1, PHD2, MSS10*) has been isolated multiple times as a gene which, when overproduced, improves iron uptake (EIDE and GUARENTE 1992), enhances pseudohyphal growth (GIMENO and FINK 1994) at least in part through activation of the *MUC1* gene (LAMBRECHTS *et al.* 1996a), ectopically activates *FUS1* transcription (RAMER *et al.* 1992), and dere-

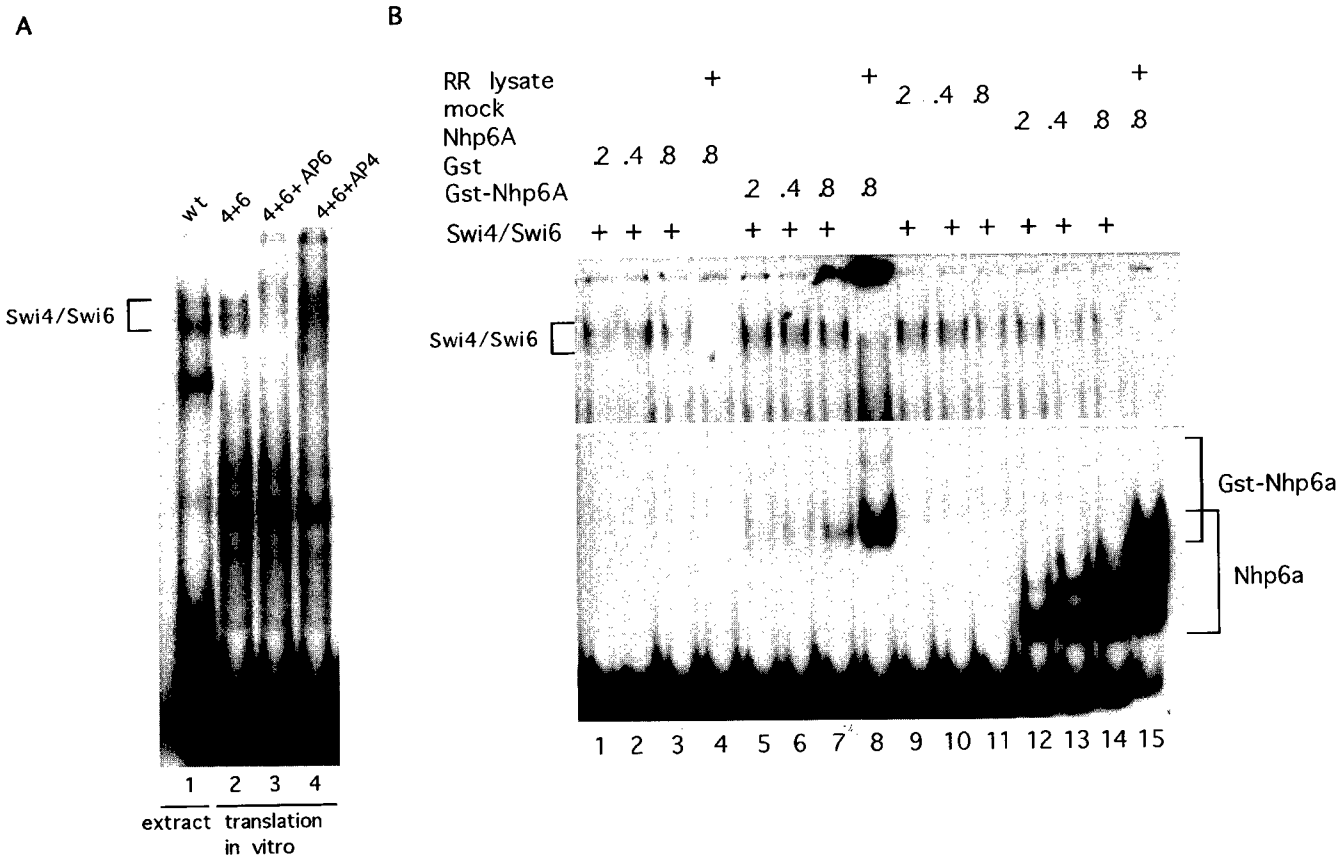

FIGURE 5.—Nhp6A does not cooperate with the Swi4/Swi6 complex in binding to the *HO* promoter. (A) An *in vitro*-translated Swi4/Swi6 complex was mixed with the labeled *HO* promoter DNA, and gel retardation analysis was performed. Lanes 2–4 show the *in vitro*-generated Swi4/Swi6 complex bound to the DNA (translation *in vitro*). In lanes 3 and 4, 2 and 5 ml of affinity-purified Swi6 (AP6) and Swi4 (AP4) antibodies, respectively, were added to the DNA-binding reactions. Lane 1 shows the Swi4/Swi6 complex generated on the *HO* promoter by wild-type yeast extract. (B) A gel retardation experiment performed with mixtures of purified Nhp6A and the *in vitro*-produced Swi4/Swi6 complex. Two exposures of one gel are shown together to follow both the abundant Nhp6A complexes and the less abundant Swi4/Swi6 complex. The reagents added are listed on the left. Plus indicates additions of either the rabbit reticulocyte lysate-translated Swi4 and recombinant Swi6 (Swi4/Swi6) or the unprogrammed lysate (RR lysate). For this experiment Gst-Nhp6A and Gst were purified from *E. coli* as indicated in MATERIALS AND METHODS. Nhp6A or a mock preparation of it was obtained by thrombin cleavage of Gst-Nhp6A or Gst immobilized on glutathione beads (see MATERIALS AND METHODS). Numbers designate the microliter amounts of purified Gst-Nhp6A, Gst, Nhp6A, or mock preparation added to the reactions. These proteins were added to the DNA-binding reactions together with reticulocyte lysate mixtures and incubated as described in MATERIALS AND METHODS. Brackets on the right show the positions of the DNA-bound Nhp6A and Gst-Nhp6A complexes and the bracket on the left shows the position of the Swi4/Swi6 complex.

presses glucoamylase genes (LAMBRECHTS *et al.* 1996b). In all of the screens where they could be measured, including ours, increased levels of *MSN1* led to activation of gene expression. It is therefore likely that Msn1 is an activator protein. Consistent with this, ESTRUCH and CARLSON (1990) have shown that, when fused to LexA, Msn1 functions as a transcriptional activator. Msn1 does not have strong DNA-binding activity of its own, suggesting that it may bind to the protein component of transcription complexes. The spectrum of action of *MSN1* seems to be rather broad and now includes *HO, FUS1, SUC2, MUC1,* and *STA1-3* genes. The fact that Gst-Msn1 overexpression is toxic to cells suggests that *MSN1* may have other targets that are critical for growth and viability, and their deregulation is deleterious for the cells. Alternatively, Gst-Msn1 could titrate out essential components of transcriptional apparatus or interfere with chromosome mechanics.

Interestingly, *PHD1*, a gene coding for a protein with homology to Swi4, was isolated in the same screen for enhancers of pseudohyphal growth in which *MSN1* was identified (as *PHD2*) (GIMENO and FINK 1994). It is plausible that Phd1 and Msn1 may cooperate in activation of genes required for pseudohyphae formation in a manner similar to the way Msn1 and Swi4 cooperate to activate *HO*. The fact that the Msn1-mediated enhancement of *ho::lacZ* transcription absolutely requires Swi4 is consistent with this possibility. However, this cooperation is unlikely to be mediated through stable interaction with Swi4/Swi6 complexes because Msn1

suppression of *HO* transcription can occur in the absence of Swi6 and does not occur at a second Swi4/Swi6-regulated gene, *CLN1*. In addition, we cannot detect physical association between Msn1 and either Swi4 or Swi6. Perhaps it is more likely that Msn1 activates transcription through other site(s) within the *HO* promoter, or it may assist the basic transcriptional machinery as a cofactor. Alternatively, it may facilitate formation of an active chromatin configuration within the *HO* promoter.

A different picture emerges in the case of *NHP6A* (KOLODRUBETZ and BURGUM 1990), which is the other suppressor of *SWI6* mutations that we have characterized. Nhp6A and its close homologue Nhp6B bear homology to the higher eukaryotic HMG1 protein family, which has been implicated in DNA replication and transcription (BUTLER *et al.* 1985; TREMETNICK and MOLLOY 1988; SINGH and DIXON 1990; GE and ROEDER 1994; STELZER *et al.* 1994; SHYKIND *et al.* 1995). *In vitro*, Nhp6A and Nhp6B have been shown to bind DNA nonspecifically but with relatively high affinity. The proteins wrap DNA in a way that introduces negative supercoils (PAULL and JOHNSON 1995). Recently Nhp6A and Nhp6B were shown to be required for inducible transcription of several messenger RNAs *in vivo*, and to facilitate formation of Tbp complexes with the TATA regions of promoters *in vitro* (PAULL *et al.* 1996). Our results indicate that the two Swi4/Swi6-regulated promoters, *HO* and *CLN1*, are also influenced by Nhp6A and Nhp6B proteins. Elevated levels of Nhp6A or Nhp6B increase *ho::lacZ* transcription in strains carrying defective but not null alleles of *SWI6*. Moreover, strains deleted for both *NHP6A* and *NHP6B* have low *HO* and *CLN1* mRNA levels that can be rescued by transformation of *NHP6A* on a high copy plasmid into these strains. This suggests that Nhp6A may cooperate with Swi4/Swi6 in transcriptional activation. However, if this is the case, the interaction is probably unstable because we have not been able to demonstrate any association between Nhp6A and Swi6, nor have we been able to detect any positive effect of Nhp6A upon the ability of the Swi4/Swi6 complex to bind DNA *in vitro*. It is equally plausible that *NHP6A* and *NHP6B* affect transcription indirectly by increasing expression of an unidentified protein that enhances Swi4/Swi6 activity or that *NHP6A* and *NHP6B* affect the chromatin conformation of the promoters. Such an activity would be undetectable *in vitro* with bandshift assays, which use naked DNA.

Massive overproduction of Nhp6A is deleterious to the cell (ESPINET *et al.* 1995 and data not shown), but does not lead to the cell cycle stage-specific arrest. The DNA-binding function of this Gst-Nhp6A fusion is intact, so its toxicity may be attributed to increased binding to genomic DNA, which might cause general inhibition or inappropriate activation of transcription. Recently the *NHP6A* gene was isolated as a high copy suppressor of *slk1(bck1)* defects and implicated as a downstream component of the Slt2/Mpk1 MAP kinase pathway that leads from Pkc1 and is involved in growth control and cell morphogenesis (COSTIGAN *et al.* 1994). *nhp6AB* deletion strains were shown to share many phenotypes with *pkc1*, *slk1(bck1)* and *slt2(mpk1)* deletion strains. The exact function of Nhp6 proteins in the Slt2 pathway has not been elucidated, but it has been proposed that they participate in Slt2-responsive transcription. We have found that Nhp6A and Nhp6B affect expression of *HO* and *CLN1*, which are controlled by the Swi4/Swi6 complex. This complex has also been implicated in transcriptional regulation of some of the Slt2-responsive genes (IGUAL *et al.* 1996). Moreover, Swi6 can be phosphorylated by Slt2 *in vitro*, and its phosphorylation state correlates with Slt2 activity *in vivo* (MADDEN *et al.* 1997). Consistent with this, *swi6* deletion strains have been shown to be more sensitive to caffeine than wild type, and they share this phenotype with *slt2* mutants (IGUAL *et al.* 1996). We have found that the *swi6-405* mutant is also sensitive to caffeine and that this phenotype can be suppressed by Nhp6A or Nhp6B expressed from a high copy plasmid (Figure 4A). All these findings are consistent with the notion that Nhp6A and Nhp6B proteins may be utilized in a similar fashion both at "classic" Swi4/Swi6-dependent promoters, *HO* and *CLN1*, as well as at the promoters that may be both Slt2 dependent and Swi4/Swi6 dependent.

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